

TITLE OF THE INVENTION

Bacterial Superantigen Vaccines

This is a continuation-in-part application which claims priority from an
 5 earlier filed application serial no. 08/882,431 filed on June 25, 1997 still
 pending, and divisional application serial no. 09/144,776 filed on September 1,
 1998 now allowed.

INTRODUCTION

Staphylococcal enterotoxins (SEs) A through E are
 10 the most common cause of food poisoning [Bergdoll,
 M.S. (1983) In Easom CSF, Aslam C., eds. Staphylococci
 and staphylococcal infections. London: Academic Press,
 pp 559-598] and are associated with several serious
 diseases [Schlievert, P.M. (1993) *J. Infect. Dis.*
 15 **167**: 997-1002; Ulrich *et al.* (1995) *Trends Microbiol.*
3: 463-468], such as bacterial arthritis [Schwab *et*
al. (1993) *J. Immunol.* **150**: 4151-4159; Goldenberg *et*
al. (1985) *N. Engl. J. Med.* **312**: 764-771], other
 autoimmune disorders [Psnettt, D. N. (1993) *Semin.*
 20 *Immunol.* **5**: 65-72], and toxic shock syndrome
 [Schlieverst, P.M. (1986) *Lancet* **1**: 1149-1150; Bohach
et al. (1990) *Crit. Rev. Microbiol.* **17**: 251-272]. The
 nonenterotoxigenic staphylococcal superantigen toxic shock
 syndrome toxin-1 (TSST-1) was first identified as a
 25 causative agent of menstrual-associated toxic shock
 syndrome [Schlievert *et al.* (1981) *J. Infect. Dis.*
143: 509-516]. Superantigen-producing *Staphylococcus*
aureus strains are also linked to Kawasaki syndrome,
 an inflammatory disease of children [Leung *et al.*
 30 (1993) *Lancet* **342**: 1385-1388].

The staphylococcal enterotoxins A-E, toxic shock
 syndrome toxin-1 (TSST-1), and streptococcal pyrogenic
 exotoxins A-C are soluble 23-29-kD proteins commonly
 referred to as bacterial superantigens (SAGs).

Bacterial superantigens are ligands for both major histocompatibility complex (MHC) class II molecules, expressed on antigen-presenting cells, and the variable portion of the T cell antigen receptor β chain (TCR V β) [Choi et al. (1989) *Proc. Natl. Acad. Sci. USA* **86**:8941-8945; Fraser, J.D. (1989) *Nature* **339**:221-223; Marrack et al. (1990) *Science* **248**: 705-711; Herman et al. (1991) *Annu. Rev. Immunol.* **9**: 745-772; Mollick et al. (1989) *Science* **244**:817-820].

Each bacterial superantigen has a distinct affinity to a set of TCR V β , and coligation of the MHC class II molecule polyclonally stimulates T cells [White et al. (1989) *Cell* **56**: 27-35; Kappler et al. (1989) *Science* **244**: 811-813; Takimoto et al. (1990) *Eur J. Immunol.* **140**: 617-621]. Pathologically elevated levels of cytokines that are produced by activated T cells are the probable cause of toxic shock symptoms [Calson et al. (1985) *Cell. Immunol.* **96**: 175-183; Stiles et al. (1993) *Infect. Immun.* **61**: 5333-5338]. In addition, susceptibility to lethal gram-negative endotoxin shock is enhanced by several bacterial superantigens [Stiles, et al., *supra*]. Although antibodies reactive with superantigens are present at low levels in human sera [Takei et al. (1993) *J. Clin. Invest.* **91**: 602-607], boosting antibody titers by specific immunization may be efficacious for patients at risk for toxic shock syndrome and the other disorders of common etiology.

A vaccine approach to controlling bacterial superantigen-associated diseases presents a unique set of challenges. Acute exposure to bacterial superantigens produces T cell anergy, a state of specific non-responsiveness [Kawabe et al. (1991)

Nature **349**: 245-248], yet T cell help is presumably a requirement for mounting an antibody response.

Presently, the only superantigen vaccines available are chemically inactivated toxoids from different bacteria which have several disadvantages. The chemical inactivation process can be variable for each production lot making the product difficult to characterize. The chemical used for inactivation, (e.g. formaldehyde), is often toxic and does not negate the possibility of reversion of the inactivated superantigen to an active form. In addition, the yields of wild-type toxin from bacterial strains used for making toxoids are often low.

SUMMARY OF THE INVENTION

The present invention relates to a vaccine which overcomes the disadvantages of the chemically inactivated toxoids described above. The superantigen vaccine(s) of the present invention is/are designed to protect individuals against the pathologies resulting from exposure to one or several related staphylococcal and streptococcal toxins. The superantigen vaccine is comprised of a purified protein product that is genetically attenuated by DNA methodologies such that superantigen attributes are absent, however the superantigen is effectively recognized by the immune system and an appropriate antibody response is produced.

Specifically, the vaccine of the present invention is a product of site-directed mutagenesis of the DNA coding sequences of superantigen toxins resulting in a disruption of binding to both the MHC class II receptor and to the T-cell antigen receptor. A comprehensive study of the relationships of the superantigen structures of TSST-1, streptococcal

pyrogenic exotoxin-A (SpeA), staphylococcal enterotoxin B (SEB), and staphylococcal enterotoxin A, to receptor binding were undertaken to provide insight into the design of the vaccine. From these studies, critical amino acid residues of the toxin responsible for binding the superantigen to the human MHC receptor were defined. Site-directed mutagenesis of the gene encoding the toxin and expression of the new protein product resulted in a superantigen toxin with disrupted binding to the MHC receptors.

Therefore, it is an object of the present invention to provide a superantigen toxin DNA fragment which has been genetically altered such that binding of the encoded altered toxin to the MHC class II or T-cell antigen receptor is disrupted. Such a DNA fragment is useful in the production of a vaccine against superantigen toxin infections.

It is another object of the present invention to provide a superantigen toxin amino acid sequence which has been altered such that the binding of the encoded altered toxin to the MHC class II or T-cell antigen receptor is disrupted. Such a sequence is useful for the production of a superantigen toxin vaccine.

It is another object of the invention to provide a recombinant vector comprising a vector and the DNA fragment described above.

It is a further object of the present invention to provide host cells transformed with the above-described recombinant DNA constructs. Host cells include cells of other prokaryotic species or eukaryotic plant or animal species, including yeasts, fungi, plant culture, mammalian and nonmammalian cell lines, insect cells and transgenic plants or animals.

It is another object of the present invention to provide a method for producing altered superantigen toxin with disrupted MHC class II and T-cell antigen receptor binding which comprises culturing a host cell under conditions such that a recombinant vector comprising a vector and the DNA fragment described above is expressed and altered superantigen toxin is thereby produced, and isolating superantigen toxin for use as a vaccine against superantigen toxin-associated bacterial infections and as a diagnostic reagent.

It is still another object of the invention to provide a purified altered superantigen toxin useful as a vaccine and as a diagnostic agent.

It is another object of the invention to provide a purified altered superantigen toxin for the therapeutic stimulation of, or other *in vivo* manipulation of, selective T cell subsets, or *ex vivo* manipulation of T cells for *in vivo* therapeutic purposes in mammals. Diseases, such as autoimmunity, wherein T-cell responses of limited diversity (oligoclonal) are evident. Altered superantigens may be used to therapeutically inactivate (induce anergy in) T cells in diseases wherein oligoclonal T-cell responses are evident such as autoimmune diseases, for example. For diseases in which specific T-cell subsets are not active or are anergic, altered superantigens may be used to therapeutically stimulate these T cells. Such diseases include, but are not limited to, infectious diseases and cancers wherein specific subsets of cytotoxic or helper T cells are inactivated or are otherwise unable to respond normally to the antigenic stimulation of the disease moiety.

It is a further object of the present invention to provide an antibody to the above-described altered

superantigen toxin for use as a therapeutic agent and as a diagnostic agent.

It is yet another object of the invention to provide a superantigen toxin vaccine comprising an
5 altered superantigen toxin effective for the production of antigenic and immunogenic response resulting in the protection of an animal against superantigen toxin infection.

It is a further object of the invention to
10 provide a multivalent superantigen toxin vaccine comprising altered toxins from a variety of streptococcal and staphylococcal toxins effective for the production of antigenic and immunogenic response resulting in the protection of an animal against
15 infection with bacterial superantigen toxin-expressing strains and against other direct or indirect exposures to bacterial superantigen toxins such as might occur by ingestion, inhalation, injection, transdermal or other means.

20 It is yet another object of the present invention to provide a method for the diagnosis of superantigen toxin-associated bacterial infection comprising the steps of:

(i) contacting a sample from an individual
25 suspected of having a superantigen toxin-associated bacterial infection with antibodies which recognize superantigen toxin using antibodies generated from the altered superantigen toxin; and

(ii) detecting the presence or absence of a
30 superantigen-associated bacterial infection by detecting the presence or absence of a complex formed between superantigen toxin in said sample and antibodies specific therefor.

It is yet another object of the present invention to provide a method for the diagnosis of superantigen bacterial infection comprising the steps of:

- (i) contacting a sample from an individual
 - 5 suspected of having the disease with lymphocytes which recognize superantigen toxin produced by said superantigen bacteria or lymphocytes which recognize altered superantigen toxin; and
- (ii) detecting the presence or absence of
 - 10 responses of lymphocytes resulting from recognition of superantigen toxin. Responses can be, for example, measured cytokine release, increase of activation markers, mitotic activity, or cell lysis. The lymphocytes responding to the altered superantigen
 - 15 toxins recognize them as recall antigens not as superantigens, therefore the response is an indicator of prior exposure to the specific superantigen. The absence of a response may indicate no prior exposure, a defective immune response or in some cases a
 - 20 manifestation of T-cell anergy. Anergy is defined here as antigen-specific or a generalized non-responsiveness of subsets of T cells.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody

- 25 against an altered superantigen toxin and ancillary reagents suitable for use in detecting the presence of superantigen toxin in animal tissue or serum.

It is another object of the present invention to provide a detection method for detecting superantigen

- 30 toxins or antibodies to superantigen toxin in samples, said method comprising employing a biosensor approach. Such methods are known in the art and are described for example in Karlsson et al. (1991) *J. Immunol. Methods* **145**, 229-240 and Jonsson et al. (1991) *Biotechniques* **11**, 620-627.
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It is yet another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of superantigen-associated bacterial infection, said method comprising providing to an individual in need of such treatment an effective amount of sera from individuals immunized with one or more altered superantigen toxins from different bacteria in a pharmaceutically acceptable excipient.

It is further another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of superantigen toxin-associated bacterial infection, said method comprising providing to an individual in need of such treatment an effective amount of antibodies against altered superantigen toxins in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of bacterial superantigen toxin infection, said method comprising providing to an individual in need of such treatment an effective amount of altered superantigen from a variety of streptococcal and staphylococcal bacteria in order to inhibit adhesion of superantigen bacterial toxin to MHC class II or T-cell receptors by competitive inhibition of these interactions.

It is yet another object of the present invention to provide a therapeutic method for the treatment of diseases that may not be associated directly with superantigen toxins but which result in specific nonresponsiveness of T-cell subsets, said method comprising the administration of altered superantigen toxins, *in vivo* or *ex vivo*, such that T-cell subsets are expanded or stimulated. Diseases which cause

anergy or nonresponsiveness of T-cells include, but are not limited to, infectious diseases.

It is another object of the present invention to provide a therapeutic method for the treatment of diseases associated with expanded or over-stimulated T-cell subsets, such as autoimmunity for example, said method comprising administration of altered superantigen toxin, *in vivo* or *ex vivo*, such that anergy or inactivation of disease associated T-cells is produced. In this case, superantigen mutants can be designed with altered but not attenuated T-cell receptor binding, to cause anergy of only the select (i.e. 1-3) T-cell subsets that are pathologically activated.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Staphylococcal and streptococcal superantigen amino acid sequence homologies, compiled with Genetics Computers Group of Univ. of Wisconsin software.

Figure 2. Comparison of mutant SEB and SEA biological activities.

A. SEB mutant HLA-DR1-binding; **B.** SEA mutant HLA-DR1-binding; **C.** T-cell recognition of SEA and SEB mutants. Binding of bacterial superantigens to cell surface DR1 was measured by laser fluorescence-activated flow cytometry. A representative experiment of three performed is shown. The mutants SEA D197N, the homologous SEB D199N, and SEA L11Y had no effect on binding or T-cell recognition, and were used for

controls. Human T-cell proliferation, assessed by [³H]thymidine incorporation, was measured in response to SEA (Y64A) or SEB (Y61A) mutants and controls that retained DR1-binding affinities. Each data point
 5 represents the mean of triplicate determinations; SEM <5%.

Figure 3. Sequence and secondary structural alignment of bacterial superantigen toxins. Analyses were performed with the applications PILEUP and
 10 PROFILE from the Computer Genetics Group (Madison, WI) using sequence data obtained from a variety of sources. Amino acid residue numbering is based on the SEA sequence.

Figure 4. Detection of TNF- α (a), IL-1 α (B), IL-
 15 6 (C) and IFN- γ (D) in the serum of mice injected with SEA (open circles), LPS (open triangles), or SEA plus LPS (open squares). Values for TNF- α and IL-1 α represent the mean of duplicate samples, with an SEM of \pm 5%. INF- γ and IL-6 values represent the mean of
 20 duplicate and triplicate samples, respectively. The SEMs for IFN- γ and IL-6 readings were \pm 5% and \pm 10%, respectively.

Figure 5. Mutant SEA vaccines that have attenuated major histocompatibility complex class II
 25 or T-cell antigen receptor binding do not induce T-cell anergy. Mice were given three doses of wild type (WT) SEA or site-specific mutant vaccine, plus adjuvant. Control animals received adjuvant alone or were untreated; 2 weeks after final injection, pooled
 30 mononuclear cells were collected from spleens of 4 mice from each group. Results are represented as mean cpm (\pm SD) of quadruplicate wells incubated with 100 ng/ml WT SEA for 72 h and then pulse-labeled for 12 h with [³H]thymidine. P<0.0001 (analysis of variance

for repeated measures comparing untreated, adjuvant, Y64A, and Y92A to WT SEA group).

Figure 6. No superantigen-induced T-cell anergy is exhibited by rhesus monkeys immunized with the vaccine B899445. Peripheral blood lymphocytes were incubated with titrated concentrations of wild-type superantigens from individual rhesus monkeys (K422 and N103) that were immunized with B899445. T-cell proliferation was assessed by [³H]thymidine incorporation. Each data point represents the mean of triplicate determinations; SEM <5%.

Figure 7. Antibody responses of rhesus monkeys immunized with a combined vaccine consisting of B899445 (SEB) and A489270 (SEA). The antibody levels were measured by ELISA, using plates coated with SEA, SEB or SEC1 as listed. Monkey G8 is a non-immunized control. SEM <5% for triplicate measurements.

Figure 8A, 8B and 8C. Biological activities of TSST-1 mutants. A, Mutations of TSST-1 at amino acid position 30 (L30R, L30A) results in greatly diminished interactions with cell surface HLA-DR, measured by laser fluorescence-activated flow cytometry and FITC-labeled rabbit anti-TSST-1 antibody (affinity purified). B, Mutations of TSST-1 at amino acid position 30 (L30R, L30A) results in greatly diminished activation of human lymphocytes; C, Introduction of an additional mutation, H135A to the TSST-1 mutant L30R results in the maximum reduction in T-cell stimulation. Human T-cell proliferation, was assessed by [³H]thymidine incorporation, using a 12 h pulse with label and harvesting cells after 60 h of culture. Each data point represents the mean of triplicate determinations; SEM <5%.

Figure 9. Antibody response to TSST-1 mutant L30R. Mice received a total of three injections of

vaccine (20 mg/mouse) in Alhydrogel, two weeks between injections. Sera were sampled two weeks after last vaccination and anti-TSST-1 specific antibody was measured by ELISA, using plates coated with wild-type TSST-1. Pooled non-immune mouse sera were used as negative control.

Figure 10A, and 10B. Biological activities of SpeA mutants. A, Mutations of SpeA at amino acid position 42 (L42R) results in greatly diminished interactions with cell surface HLA-DR, measured by laser fluorescence-activated flow cytometry and FITC-labeled rabbit anti-SpeA antibody (affinity purified). B, Mutations of SpeA at amino acid position 42 (L42R or L42A) results in greatly diminished activation of human lymphocytes. Human T-cell proliferation, was assessed by [³H]thymidine incorporation, using a 12 h pulse with label and harvesting cells after 60 h of culture. Each data point represents the mean of triplicate determinations; SEM <5%.

Figure 11. Mouse antibody response to SpeA L42R and SpeA-B fusion constructs. BALB/c mice were vaccinated three times with 10 µg plus adjuvant (MPL™ + TDM+ CWS Emulsion, RIBI ImmunoChem Research, Inc., Hamilton, MT) of each construct, allowing two weeks between injections. Sera from each experimental group (n=5) were pooled for measurement of specific antibodies. Data shown are antigen-specific antibodies (ELISA units) present in a 1:100,000 dilution of pooled sera from mice vaccinated with SpeA L42R, SpeA-B fusion or adjuvant only.

Figure 12. T-cell response in vitro of mononuclear cells from transgenic mice expressing HLA-DQ8αβ and human CD4 closely approximate the physiological response of humans. Mononuclear cells were isolated from spleens of transgenic mice

expressing HLA-DR3, HLA-DQ8 or HLA-DR2 β /IE α , or non-transgenic BALB/c mice and human peripheral blood (1×10^5 /well). Following 60 h culture with SpeA, cells were pulse-labeled (12 h) with 1 uCi of [3 H]thymidine. DNA from cells was harvested onto fiberglass filters and incorporated radioactivity measured by liquid scintillation.

DETAILED DESCRIPTION

The present invention relates in part to a vaccine against superantigen toxin-associated bacterial diseases. The superantigen vaccines used in this study were developed by engineering changes in the receptor-binding portions of superantigen toxins to reduce receptor-binding affinities and toxicity while maintaining antigenicity.

Five different superantigen vaccines are described in this application: staphylococcal enterotoxin A, staphylococcal enterotoxin B, staphylococcal enterotoxin C1, toxic-shock syndrome toxin-1, and streptococcal pyrogenic exotoxin-A. For each of the superantigen toxins above, a comprehensive study of the relationships of the toxin protein structure to receptor binding was undertaken to provide insight into the design of the vaccine. The study employed site-directed mutagenesis of toxin and receptor molecules, molecular modeling, protein structure and binding studies. Following these studies, toxins were altered by site-directed mutagenesis to attenuate MHC class II binding and biological activity to an essentially non-specific level. The engineered vaccines were evaluated at each stage of analysis to determine mouse and human T-cell reactivities *in vitro*, serological responses and toxicity in mice and monkeys.

In one embodiment, the present invention relates to an altered superantigen toxin protein having an amino acid sequence which has been altered such that the binding of the toxin to the MHC class II receptor is disrupted.

Comparison of amino acid sequences (**Fig. 1**) suggested that bacterial superantigens fall into groups consisting of (1) SEA, SED and SEE, (2) SEB, staphylococcal enterotoxins C1-C3 (SEC1-3), the streptococcal pyrogenic exotoxins A (SPE-A) and C (SPE-C), (3) TSST-1 and (4) the exfoliative toxins (ETA, ETB) and streptococcal pyrogenic exotoxin B (SPE-B), which are the most distant from the others in sequence. Although not available to the inventor when the inventions were first conceived and proof of principle was obtained, the x-ray crystallographic structures of several bacterial superantigens are now known. Diverse superantigens, such as SEB and TSST-1, appear to have little sequence in common, yet they exhibit homologous protein folds composed largely of β strands [Prasad, G.S. et al. (1993) *Biochemistry* **32**, 13761-13766; Acharya, R.K. et al. (1994) *Nature* **367**, 94-97; Swaminathan, S. et al. (1992) *Nature* **359**, 801-806] within two distinct domains. Differences between the proteins are located primarily in highly variable regions comprised of several surface loops, such as the disulfide-bonded loop which is absent from TSST-1 and at the amino terminus.

The X-ray crystal structures of SEB and TSST-1 complexed with HLA DR1 are known [Kim, J. et al. (1994) *Science* **266**, 1870-1874 ; Jardetzky, T.S. et al. (1994) *Nature* **368**, 711-718]. The region of HLA DR1 that contacts SEB consists exclusively of α subunit surfaces. The main regions of SEB involved are two conserved sites: a polar pocket derived from three β

strands of the β barrel domain and a highly solvent-exposed hydrophobic reverse turn. The polar binding pocket of SEB contains a glutamate and two tyrosines that accommodate Lys39 of the α subunit of HLA DR1, while the hydrophobic region consists of a leucine and flanking residues that make several contacts with the HLA DR α chain. The HLA DR1 binding sites of both TSST-1 and SEB overlap significantly. The hydrophobic binding contacts of other SAg with the HLA DR α chain have been proposed [Ulrich, *et al.* (1995). *Nature, Struct. Biol* **2**, 554-560] to be similar to those found in SEB and TSST-1. A motif consisting of a leucine in a reverse turn [Ulrich *et al.* (1995), *supra*] is conserved among bacterial superantigens and may provide the key determinant (hydrophobic or otherwise) for binding HLA-DR. However, TSST-1 does not have a highly charged residue in the polar pocket that interacts with Lys39 of the HLA DR α chain and uses an alternative conformational binding mode that allows TSST-1 to interact with HLA DR1 β -chain residues and the carboxy-terminal region of the antigenic peptide.

Both SEA and SEE bind to the β subunit of DR by means of a single zinc atom [Fraser, J.D. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5507-5511]. The amino-terminal domain of SEA interfaces with the HLA DR α chain [Ulrich, *et al.* (1995)], while SEA C-terminal domain residues His187, His225 and Asp227 form a zinc-coordination complex, likely with His-81 from the β chain of an adjoining HLA DR molecule. However, our results have shown that binding of superantigen to the HLA DR β subunit does not directly stimulate T cells [Ulrich *et al.* (1995) *Nature, Struct. Biol.* **2**, 554-560], but increases the potential of the bound SEA to interact with the α chain of

another HLA DR, thus increasing the biological potency.

A least-squares superimposition of the unbound molecules of modeled SEA and the crystal structure of SEB, aligned according to their structurally conserved α -helical and β -strand regions, exhibited a global folding pattern which is very similar. Differences between the two structures are calculated to be located primarily in loops of low sequence homologies, with the largest positional deviations occurring between structurally conserved regions of residues 18-20, 30-32, 173-181, 191-194, and the cysteine-loop region (90-111). Only one of these regions in SEB makes significant contact (residue Y94 [Y=tyrosine] in particular) with the HLA-DR1 molecule [Jardetzky, T.S. et al. (1994) *Nature* **368**, 711-718].

The binding interface between SEB and HLA-DR1 consists principally of two structurally conserved surfaces located in the N-terminal domain: a polar binding pocket derived from three β -strand elements of the β -barrel domain and a hydrophobic reverse turn. The binding pocket of SEB contains residues E67 (E=Glutamic acid), Y89 (Y=Tyrosine) and Y115 (Y=tyrosine), and binds K39 (K=Lysine) of the DR α subunit. The amino acid one letter code is defined as the following: A= Alanine (Ala), I= Isoleucine (Ile), L= Leucine (Leu), M= Methionine (Met), F= Phenylalanine (Phe), P= Proline (Pro), W=Tryptophan (Trp), V=Valine (Val), N= Asparagine (Asn), C=Cysteine (Cys), Q= Glutamine (Q), G= Glycine (Gly), S= Serine (Ser), T= Threonine (Thr), Y= Tyrosine (Tyr), R= Arginine (Arg), H=Histidine (His), K= Lysine (Lys), D= Aspartic acid (Asp), and E= Glutamic acid (Glu). For SEA, the binding interface with the DR molecule is modeled to contain a similar binding pocket consisting

of residues D70, Y92 and Y108. Mutation of residue Y89 in SEB or Y92 in SEA to alanine (**Fig. 2**) resulted in greater than 100-fold reduction in DR1 binding. The substitution of alanine for Y89 in SEB and Y92 in SEA eliminates the hydrogen bond with K39 and disrupts packing interactions with adjacent protein residues. Modeling of the SEA mutant Y92A predicts an increase in solvent-accessible surface area for Y108 by a factor of two greater than the wild-type structure, allowing the formation of a hydrogen bond to the carboxylate group of D70 and thus disrupting key anchoring and recognition points for HLA-DR1. This effect is expected to be somewhat less in SEB due to the longer side chain at E67. Substitution of SEB Y115 with alanine also resulted in greater than 100-fold reduction of binding. In contrast, the same replacement of Y108 in SEA yielded little to no change in DR1 binding (**Fig. 2a**), suggesting the primary importance of SEA residues Y92 and D70 for stabilizing interactions with K39. The K39 side chain of DR α forms a strong ion-pair interaction with the SEB E67 carboxylate group and hydrogen bonds with the hydroxyl groups of Y89 and Y115. Substitution of SEB E67 by glutamine reduced binding affinity by greater than 100-fold (**Fig. 2**), reflecting the replacement of the strong ionic bond with a weaker hydrogen bond. To optimize ion-pair interactions of the analogous SEA site, the shorter carboxylate side chain of D70 is predicted to shift K39 of DR α , weakening interactions with SEA Y108. The substitution of alanine for SEA Y108 is thus more easily accommodated than the homologous substitution of SEB Y115, without loss in DR1 binding.

Comparisons of the polar pocket with other bacterial superantigens were then made. SEC1-3 and

SPE-A have conserved the critical DR1 binding-interface residues (**Fig. 1**), and share with SEB and SEA secondary structural elements of the DR1-binding surfaces. Asparagine in SED (N70) replaces the acidic side chain present in SEA, SEB, SPE-A and SEC1-3. Accordingly, for SED the salt bridge of the polar pocket is likely to be replaced by a hydrogen bond. Overall, DR1 affinities for SED and SEA appeared to be equivalent (**Fig. 2b**), indicating that other interactions may compensate for the absence in SED of the ion-pair found in the other superantigens. For the case of TSST-1, mutating DR α residues K39 to serine or M36 to isoleucine has been shown to greatly reduce binding [Panina-Bordignon *et al.* (1992) *J. Exp. Med.* **176**: 1779-1784]. Although primarily hydrophobic, the critical TSST-1 structural elements are conserved with the SEA and SEB polar binding pocket. SEB residues Y89 and Y115 are homologous to T69 and I85 in TSST-1, respectively, and SEB E67 is replaced by I46. These TSST-1 residues are positioned in a conserved β -barrel domain found in both SEB and SEA. However, the TSST-1 site lacks polarity equivalent to SEB/SEA, and hydrogen bonding with the hydroxyl of TSST-1 residue T69 would require that DR α K39 extend 5 Å into the pocket. TSST-1 binding utilizes an alternative strategy [Kim *et al.* (1994) *Science* **266**:1870-1874] consisting of hydrophobic contacts centered around residue I46, and potential ionic or hydrogen bonds bridging DR α residues E71 and K67 to R34 and D27, respectively, of TSST-1.

The hydrophobic region of the binding interface between SEB and the HLA-DR1 molecule consists of SEB residues 44-47, located in a large reverse turn connecting β -strands 1 and 2 of SEB. These residues appear to make strong electrostatic interactions with

DR α through their backbone atoms. The mutation of L45 to an arginine reduced overall HLA-DR1 binding greater than 100-fold (**Fig. 2b**), attributable to the less energetically favorable insertion of a highly charged residue into a hydrophobic depression on the DR1 molecule. The modeled DR1-SEA complex presents similar interactions with the SEA backbone atoms, with the exception of a glutamine (Q49) replacing SEB Y46. Mutation of L48 to glycine in SEA (homologous to L45 of SEB) has been reported to decrease T-cell responses. SEB L45 and the comparable L30 of TSST-1 are the most extensively buried residues in the DR1 interface. The leucine is conserved among the bacterial superantigens (**Fig. 3**) and may provide the necessary hydrophobic structural element for surface complementarity with DR1, consistent with the mutagenesis data for SEB and SEA.

The inventor has performed similar structure and function studies with TSST-1, SEC1 and SPE-A.

In determining the overall affinity of the superantigen for DR1, a contributory role is played by structural variations around the common binding motifs. A short, variable structured, disulfide-bonded loop is found in SEA and a homologous longer loop in SEB. The SEB residue Y94, contained within this loop, forms hydrophobic interactions with L60 and A61 of the DR α subunit. Replacement of Y94 with alanine partially inhibits DR1 binding (**Fig. 2a,b**). An alanine is found in SEA (A97) and SEE at the position equivalent to SEB Y94, and mutating this residue in SEA to tyrosine results in disrupted instead of stabilized interactions with DR1 (**Fig. 2a**). Although the disulfide loops differ in structure between SEA and SEB, A97 apparently contributes to the DR α binding interface in a manner similar to Y94 of

SEB. Because TSST-1 lacks a disulfide loop, similar contacts with DR α are replaced by interactions with β -strands of TSST-1. In a like manner, the absence of a salt bridge between the residues K39 of DR α and N65 of SED is apparently compensated for by stabilizing interactions occurring outside of the otherwise conserved dominant binding surfaces (**Fig. 2a**).

The amino acid residues in contact with TCR are located in regions of high sequence variability, presenting a unique surface for interaction with the TCR. Residues implicated in TCR interactions by mutagenesis of SEA and SEB reside in variable loop regions, while TSST-1 mutants that affect TCR binding are mainly located in an α helix [Acharya, R.K. et al. (1994) *Nature* **367**, 94-97; Kim, J. et al. (1994) *Science* **266**, 1870-1874]. Specifically, mutations that diminish T-cell receptor recognition of SEB include residues N23, Y61, and the homologous SEA N25 or Y64 (**Fig. 2c**). SEA residues S206 and N207 also control T-cell responses [Hudson, et al. (1992) *J. Exp. Med.* **177**: 175-184]. Mutants of the polar binding pocket, SEA Y92A and SEB Y89A, equivalently reduced T-cell responses (**Fig. 2c**), reflecting the observed decreases in DR1-binding (**Fig. 2a, b**). While supporting reduced T-cell responses, mutants SEA Y64A and SEB Y61A retained normal affinities for DR1 (**Fig. 2a-c**).

In view of the detailed description of the present invention and the results of molecular modelling and structural studies of staphylococcal and streptococcal superantigen toxins discussed above, any amino acid sequence derived from a superantigen toxin can be altered. Sequences of several superantigen toxins are already known and available to the public in sequence databases such as GenBank, for example.

The superantigen toxin sequence is preferably altered
 at the hydrophobic loop or polar binding pocket
 depending on the superantigen. Alternatively,
 residues adjacent to the hydrophobic loop or polar
 5 binding pocket that contact HLA-DR or residues at
 sites that can indirectly alter the structure of the
 hydrophobic loop or polar pocket can be altered. The
 number of residues which can be altered can vary,
 preferably the number can be 1-2, more preferably 2-3,
 10 and most preferably 3-4, or more with the limitation
 being the ability to analyze by computational methods
 the consequences of introducing such mutations. The
 residues which can be altered can be within 5 amino
 acid residues of the central Leucine of the
 15 hydrophobic loop (such as L45 of SEB), or within 5
 residues of one of the amino acid residues of the
 polar binding pocket that can contact HLA-DR, (such as
 E67, Y89, or Y115 of SEB), more preferably, within 3
 amino acid residues of the central Leucine of the
 20 hydrophobic loop (such as L45 of SEB), or within 3
 residues of one of the amino acid residues of the
 polar pocket that can contact HLA-DR, (such as E67,
 Y89, or Y115 of SEB), and most preferably, the central
 Leucine of the hydrophobic loop (such as L45 of SEB),
 25 or one of the amino acid residues of the polar binding
 pocket that can contact HLA-DR, (such as E67, Y89, or
 Y115 of SEB). The residues can be changed or
 substituted to alanine for minimal disruption of
 protein structure, more preferably to a residue of
 30 opposite chemical characteristics, such as hydrophobic
 to hydrophilic, acidic to neutral amide, most
 preferably by introduction of a residue with a large
 hydrated side chain such as Arginine or Lysine. In
 addition, side chains of certain nonconserved
 35 receptor-binding surfaces, can also be altered when

designing superantigen toxins with low binding affinities. These residues can include Y94 of SEB and structurally equivalent residues of other superantigens, such as A97 of SEA, or any side chain within 5 residues from these positions or any side chain in discontinuous positions (discontinuous positions are defined as amino acid residues that fold together to form part of a discrete three-dimensional structural unit but are not present on the same secondary structural unit e.g. α helix or β -strand) such as disulfide-bonded side chains, that involve, directly or indirectly, the nonconserved receptor contact surfaces outside of the polar binding pocket or hydrophobic loop. Further, amino acid residues involved with protein folding or packing can be altered when designing superantigen toxins with low binding affinities [Sundstrom et al. (1996) *EMBO J.* **15**, 6832-6840; Sundstrom et al. (1996) *J. Biol. Chem.* **271**, 32212-32216; Acharya et al. (1994) *Nature* **367**, 94-97; Prasad et al. (1993) *Biochem.* **32**, 13761-13766; Swaminathan et al. (1992) *Nature* **359**, 801-806]. Furthermore, especially for superantigens with higher affinities for T-cell antigen receptors, side chains of amino acids within 5 residues of the position represented by N23 (conserved residue in most superantigens) , N60 (conserved Asn or Trp in most superantigens) Y91 (semiconserved hydrophobic residues Trp, Ile, Val, His in most superantigens) and D210 of SEB (conserved Asp in most superantigens) can be altered when designing superantigen toxins with low binding affinities. These residues are likely to form part of the integral molecular surfaces that are in contact with T-cell antigen receptors. Because the T-cell receptor contact areas of superantigen toxins are essential for causing specific activation or

inactivation of T-cell subsets, altering residues that are unique to each superantigen but that are located within 5 residues of the positions represented by N23, N60 and Y91 can produce superantigens that affect a smaller number (e.g. 1-3) of subsets. Such altered superantigen toxins can be useful as therapeutic agents.

In another embodiment, the present invention relates to a DNA or cDNA segment which encodes a superantigen toxin such as SEA, SEB, SEC-1, SpeA, and TSST-1 to name a few, the sequence of which has been altered as described above to produce a toxin protein with altered binding ability to MHC Class II and/or T-cell receptors. For SEA, the following three mutations were introduced into the toxin molecule: Tyrosine at amino acid position 92 changed to alanine; Aspartic acid at amino acid position 70 changed to arginine; Leucine at amino acid position 48 changed to arginine. The reduction in binding to HLA DR is additive per mutation, though one or two mutations can produce a vaccine and a combination of all three mutations in one molecule produces a better vaccine. Other substitutions can also result in reduced binding.

The B899445 vaccine consists of the following three mutations simultaneously introduced into the toxin molecule: tyrosine at amino acid position 89 changed to alanine; tyrosine at amino acid position 94 changed to alanine; leucine at amino acid position 45 changed to arginine. The altered superantigen toxins can be expressed either as a full-length propeptide or as a polypeptide in which the leader peptide has been deleted. The full-length expressed product (SEA vaccine, A489270P; SEB vaccine B899445P, B2360210P) is secreted into the periplasmic space of

E. coli host cells, and the leader peptide is recognized and cleaved by a native bacterial enzymatic mechanism. The altered superantigen toxins in which the leader peptide has been deleted (A489270C, B899445C), the first residue of the mature protein is encoded by the transcriptional start site and codon for methionine (ATG), and the protein is expressed as a nonsecreted product within the host *E. coli* cell. For the TSST-1 vaccine TST30, the leucine at position 30 was changed to arginine. For the SEC1 vaccine, SEC45, the leucine at position 45 was changed to arginine. For the SPE-A vaccine, SPEA42, the leucine at position 42 was changed to arginine.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid such as any broad host range expression vector for example pUC18/19, pSE380, pHIL, pET21/24 and others known in the art. The DNA sequence is preferably functionally linked to a promoter such that the gene is expressed when present in an expression system and an altered superantigen toxin is produced. The expression system can be an *in vitro* expression system or host cells such as prokaryotic cells, or *in vivo* such as DNA vaccines.

In a further embodiment, the present invention relates to host cells stably or transiently transformed or transfected with the above-described recombinant DNA constructs. The host can be any eukaryotic or prokaryotic cell including but not limited in *E. coli* DH5 α or BL21. The vector containing the altered superantigen toxin gene is expressed in the host cell and the product of the altered toxin gene, whether a secreted mature protein

or a cytoplasmic product, can be used as a vaccine or as a reagent in diagnostic assays or detection methods, or for therapeutic purposes. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular

5 Cloning; A Laboratory Manual (1982) or DNA Cloning,
 Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG molecule, an adjuvant, a carrier, or an agent for aid
 10 in purification of altered toxin. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a
 15 source of the altered toxin described above.

A recombinant or derived altered superantigen toxin is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or
 20 expression of a recombinant expression system. In addition the altered toxin can be fused to other proteins or polypeptides for directing transport for example into the periplasm or for secretion from the cell. This includes fusion of the recombinant or
 25 derived altered superantigen to other vaccines or sequences designed to aid in purification, such as His-tagged, epitope-tagged or antibody Fc-fusions.

In a further embodiment, the present invention relates to a method of producing altered superantigen
 30 toxin which includes culturing the above-described host cells, under conditions such that the DNA fragment is expressed and a superantigen toxin protein is produced. The superantigen toxin can then be isolated and purified using methodology well known in

the art such as immunoaffinity chromatography or preparative isoelectric focusing. However, the method of purification is not critical to the performance of the vaccine. The altered superantigen toxin can be
5 used as a vaccine for immunity against infection with bacterial superantigen toxins or as a diagnostic tool for detection of superantigen toxin-associated disease or bacterial infection. The transformed host cells can be used to analyze the effectiveness of drugs and
10 agents which affect the binding of superantigens to MHC class II or T-cell antigen receptors. Chemically derived agents, host proteins or other proteins which result in the down-regulation or alteration of expression of superantigen toxins or affect the
15 binding affinity of superantigen toxins to their receptors can be detected and analyzed. A method for testing the effectiveness of a drug or agent capable of altering the binding of superantigen toxins to their receptors can be for example computer-aided
20 rational design or combinatorial library screening, such as phage display technology.

In another embodiment, the present invention relates to antibodies specific for the above-described altered superantigen toxins. For instance, an
25 antibody can be raised against the complete toxin or against a portion thereof. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the altered superantigens of the present invention, or a unique
30 portion of the altered superantigen. Materials and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986). The antibodies can be used in diagnostic assays for

detection of superantigen toxin-associated infection. Neutralizing antibodies can be used in a therapeutic composition for the treatment of amelioration of anergy and/or for the treatment of a superantigen toxin-associated infection.

In a further embodiment, the present invention relates to a method for detecting the presence of superantigen-associated bacterial infections in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of the altered superantigen described above, and contacting it with the serum of a person suspected of having a superantigen-associated bacterial infection. The presence of a resulting complex formed between the altered superantigen toxin and antibodies specific therefor in the serum can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of superantigen-associated bacterial infections.

In yet another embodiment, the present invention relates to a method for detecting the presence of superantigen toxin in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), antibodies specific for altered superantigen toxin, and contacting it with serum or tissue sample of a person suspected of having superantigen-associated bacterial infection. The presence of a resulting complex formed between toxin in the serum and antibodies specific

therefor can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of
5 superantigen-associated bacterial infection or disease such as food poisoning and toxic-shock syndrome or the detection of superantigen toxin in food and drink.

In another embodiment, the present invention relates to a diagnostic kit which contains altered
10 superantigen toxin from a specific bacteria or several different superantigen toxins from bacteria and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence of antibodies to superantigen toxin-associated bacteria
15 in serum or a tissue sample. Tissue samples contemplated can be avian, fish, or mammal including monkey and human.

In yet another embodiment, the present invention relates to a vaccine for protection against
20 superantigen toxin-associated bacterial infections. The vaccine can comprise one or a mixture of individual altered superantigen toxins, or a portion thereof. When a mixture of two or more different altered superantigen toxin from different bacteria is
25 used, the vaccine is referred to as a multivalent bacterial superantigen vaccine. The vaccine is designed to protect against the pathologies resulting from exposure to one or several related staphylococcal and streptococcal toxins. In addition, the protein or
30 polypeptide can be fused or absorbed to other proteins or polypeptides which increase its antigenicity, thereby producing higher titers of neutralizing antibody when used as a vaccine. Examples of such proteins or polypeptides include any adjuvants or

carriers safe for human use, such as aluminum hydroxide.

The staphylococcal enterotoxin (SE) serotypes SEA, SED, and SEE are closely related by amino acid sequence, while SEB, SEC1, SEC2, SEC3, and the streptococcal pyrogenic exotoxins B share key amino acid residues with the other toxins, but exhibit only weak sequence homology overall. However, there are considerable similarities in the known three-dimensional structures of SEA, SEB, SEC1, SEC3, and TSST-1. Because of this structural similarity, it is likely that polyclonal antibodies obtained from mice immunized with each SE or TSST-1 exhibit a low to high degree of cross-reaction. In the mouse, these antibody cross-reactions are sufficient to neutralize the toxicity of most other SE/TSST-1, depending upon the challenge dose. For example, immunization with a mixture of SEA, SEB, TSST-1 and SpeA was sufficient to provide antibody protection from a challenge with any of the component toxins, singly or in combination.

The likelihood of substantial antigen-cross-reactivity suggests that it may be possible to obtain immune protection for other (or perhaps all) staphylococcal superantigens by use of a minimal mixed composition of vaccines. For the case of staphylococcal superantigens, a combination of the component vaccines from SEA, SEB, SEC-1 and TSST-1 should be sufficient to provide immune protection against SEA, SEB, SEC1-3, and TSST-1. The addition of SpeA component to the trivalent mixture will allow for sufficient protection against the streptococcal toxins SpeA and SPEc. Therefore, a multivalent vaccine consisting of the altered superantigen toxins from SEA, SEB, SEC-1, TSST-1, and SpeA as described above,

is predicted to provide protective immunity against the majority of bacterial superantigen toxins.

The vaccine can be prepared by inducing expression of a recombinant expression vector
 5 comprising the gene for the altered toxin described above. The purified solution is prepared for administration to mammals by methods known in the art, which can include filtering to sterilize the solution, diluting the solution, adding an adjuvant and
 10 stabilizing the solution. The vaccine can be lyophilized to produce a vaccine against superantigen toxin-associated bacteria in a dried form for ease in transportation and storage. Further, the vaccine may be prepared in the form of a mixed vaccine which
 15 contains the altered superantigen toxin(s) described above and at least one other antigen as long as the added antigen does not interfere with the effectiveness of the vaccine and the side effects and adverse reactions, if any, are not increased
 20 additively or synergistically. Furthermore, the vaccine may be administered by a bacterial delivery system and displayed by a recombinant host cell such as *Salmonella* spp, *Shigella* spp, *Streptococcus* spp. Methods for introducing recombinant vectors into host
 25 cells and introducing host cells as a DNA delivery system are known in the art [Harokopakis *et al.* (1997) *Infect. Immun.* **65**, 1445-1454; Anderson *et al.* (1996) *Vaccine* **14**, 1384-1390; Medaglini *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6868-6872].
 30 The vaccine may be stored in a sealed vial, ampule or the like. The present vaccine can generally be administered in the form of a liquid or suspension. In the case where the vaccine is in a dried form, the vaccine is dissolved or suspended in sterilized
 35 distilled water before administration. Generally, the

vaccine may be administered orally, subcutaneously, intradermally or intramuscularly but preferably intranasally in a dose effective for the production of neutralizing antibody and protection from infection or disease.

In another embodiment, the present invention relates to a method of reducing superantigen-associated bacterial infection symptoms in a patient by administering to said patient an effective amount of anti-altered superantigen toxin antibodies, as described above. When providing a patient with anti-superantigen toxin antibodies, or agents capable of inhibiting superantigen function to a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds which is in the range of from about 1pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

In a further embodiment, the present invention relates to a therapeutic method for the treatment of diseases that may not be associated directly with superantigen toxins but which result in specific nonresponsiveness of T-cell subsets or detection of abnormally low level of subsets in peripheral blood, said method comprising the administration of altered superantigen toxins, *in vivo* or *ex vivo*, such that T-cell subsets are expanded or stimulated. Diseases which cause anergy or nonresponsiveness of T-cells include, but are not limited to, infectious diseases and cancers. The desired clinical outcome such as an increase in detectable T cell subsets or in stimulation *ex vivo* of T-cells through their antigen

receptors, such as by antigen or anti-CD3 antibody can be measured by standard clinical immunology laboratory assays.

In yet another embodiment, the present invention relates to a therapeutic method for the treatment of diseases associated with expanded or over-stimulated T-cell subsets, such as autoimmunity for example, said method comprising administration *in vivo* or *ex vivo*, of superantigen toxin altered in such a manner that only limited (1-3) T-cell subsets are stimulated but that MHC class II binding affinity still remains, such that anergy or inactivation of T-cells is produced. The desired clinical outcome can be measured as a reduction of circulating blood T-cells of the targeted subset(s) or diminished antigen or other antigen receptor-mediated-stimulatory responses by assays known in the art.

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

The following Materials and Methods were used in the Examples that follow.

Structural comparisons

Primary protein structure data are available for several bacterial superantigens, including SEA, SED, SEB, SEC1-3, TSST-1. Superantigens for which structures were unavailable were modeled using comparative techniques (HOMOLOGY program; Biosym Technologies, Inc., San Diego, CA). Before x-ray crystallography data was available, SEA was modeled by

using this method, and the model was in very close agreement with the experimentally determined structure. As an example, the amino acid sequence for SEA was aligned with the known structure of free and HLA-DR1 bound SEB, and the SEA molecule was built for both free and DR1-bound proteins. Loop segments of SEA were generated by a *de novo* method. Refinement of the modeled structures was carried out by means of molecular-dynamics simulations (DISCOVER, Biosym).

The constructed free SEA molecule was immersed in a 5-Å layer of solvent water and the α -carbon atoms lying in the structurally conserved regions were tethered to their initial positions during the simulations. For the bound SEA molecule, simulations were carried out by constructing an active-site region composed of part of the SEA molecule and the DR1 molecule inside a 10-Å interface boundary, as derived from the crystal structure of the DR1-SEB complex. Amino acid residues lying in the outer boundary were rigidly restrained at their initial positions. The active-site region was immersed in a 5-Å layer of water. Protein interactions were modeled by employing the consistent valence force field with a non-bonded cutoff distance of 11.0 Å. Simulations were initiated with 100 cycles of minimization using a steepest descent algorithm followed by 100-ps relaxation (using a 1.0 fs timestep). Structural comparisons between SEB, SEC1, and TSST-1 were performed by using the crystal structures (Brookhaven data holdings) aligned according to common secondary structural elements and/or by sequence and structural homology modeling.

Site-specific mutagenesis

Site-specific mutagenesis was performed according to the method developed by Kunkel, using gene templates isolated from *Staphylococcus aureus* strains

expressing SEA (FDA196E, a clinical isolate, Fraser, J.D. (1994) *Nature* **368**: 711-718), SEB (14458, clinical isolate), SEC1 (Toxin Technologies, Sarasota, FL), TSST-1 (pRN6550 cloned product, a clinical isolate, Kreiswirth, B. N. et al. (1987) *Mol. Gen. Genet.* **208**, 84-87), and SpeA (Toxin Technologies), respectively. Modified T7 polymerase (Sequenase, U.S. Biochemical Corp., Cleveland, OH) was used to synthesize second-strand DNA from synthetic oligonucleotides harboring the altered codon and single-stranded, uracil-enriched M13 templates. Mutagenized DNA was selected by transforming *E. coli* strain JM101. Alternatively, double stranded DNA was used as template for mutagenesis. Mutagenized sequences were confirmed by DNA sequencing (Sanger et al., 1977, *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467; Sambrook et al., 1989) using synthetic primers derived from known sequences, or universal primers. The complete coding sequences were inserted into expression plasmids such as pUC19, pSE380 or pET21 for production in *E. coli* hosts.

Protein purifications

The appropriate *E. coli* hosts were transformed with plasmids harboring the mutant toxin genes. In general, the bacteria were grown to an A600 0.5-0.6 in Terrific Broth (Difco Laboratories, Detroit, MI) containing 50 µg/mL ampicillin or kanamycin. Recombinant proteins were induced with isopropyl-β-D-thio-galactopyranoside (Life Technologies, Gaithersburg, MD) and recovered as cytoplasmic or bacterial periplasmic secretion products. Bacteria were collected by centrifugation, washed with 30 mM NaCl, 10 mM TRIS (pH 7.6), and pelleted by centrifugation and either lysed or osmotically shocked for collection of secreted proteins. Preparations

were isolated by CM Sepharose ion-exchange chromatography, rabbit antibody (Toxin Technologies, Sarasota, FL) affinity columns, ion exchange HPLC or similar methods. In some cases partially purified
 5 superantigen was further purified by preparative isoelectric focusing (MinipHor; Rainin Instrument Company, Inc., Woburn, MA.). The MinipHor was loaded with the SEA-enriched fraction from CM Sepharose chromatography in a solution containing 10% (v/v)
 10 glycerol and 1% (v/v) pH 6-8 ampholytes (Protein Technologies, Inc., Tucson, AZ). The protein preparations were allowed to focus until equilibrium was reached (approximately 4 hr, 4°C). Twenty focused fractions were collected and aliquots of each were
 15 analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The SEA-containing fractions were pooled, and refocused for an additional 4 h. The fractions containing purified SEA were pooled and dialyzed first against 1 M NaCl (48 h, 4°C)
 20 to remove ampholytes, and then against PBS (12 h, 4°C). Legitimate amino-terminal residues were confirmed by protein sequencing. Precise measurements of protein concentrations were performed by immunoassay using rabbit antibody affinity-purified
 25 with the wild-type superantigens and by the bicinchoninic acid method (Pierce, Rockford, IL) using wild-type protein as standards. All protein preparations were >99% pure, as judged by SDS-PAGE and Western immunoblots. In some cases, as when used for
 30 lymphocyte assays, bacterial pyrogens were removed by passing the protein preparations over Polymyxin B affinity columns.

Binding of superantigens to HLA-DR1

The DR1 homozygous, human B-lymphoblastoid cell
 35 line LG2 or L cells transfected with plasmids encoding

HLA-DR1 $\alpha\beta$ were used in the binding experiments. Cells were incubated 40 min (37°C) with wild-type or mutant superantigen in Hanks balanced salt solution (HBSS) containing 0.5% bovine serum albumin. The cells were washed with HBSS and then incubated with 5 μ g of specific rabbit antibody (Toxin Technology, Sarasota, FL) for 1 h on ice. Unbound antibody was removed, and the cells were incubated with FITC-labelled goat anti-rabbit IgG (Organon Teknika Corp., Durham, N.C.) on ice for 30 min. The cells were washed and analyzed by flow cytometry (FACScan; Becton Dickinson & Co., Mountain View, CA). Controls consisted of cells incubated with affinity purified anti-toxin and the FITC labelled antibody without prior addition of superantigen.

Lymphocyte proliferation

Human peripheral blood mononuclear cells were purified by Ficoll-hypaque (Sigma, St. Louis, MO) buoyant density gradient centrifugation. Genes encoding the human MHC class II molecules DR1 $\alpha\beta$ (DRA and DRB1*0101 cDNA [Bavari and Ulrich (1995) *Infect. Immun.* **63**, 423-429] were cloned into the eukaryotic expression vector pRC/RSV (Invitrogen, Carlsbad, CA), and mouse L cells were stably transfected. The transfectants were selected by fluorescence-activated cell sorting (EPICS C, Coulter Corp., Hialeah, FL) using rabbit anti-DR $\alpha\beta$ antisera and FITC-goat anti-rabbit IgG, to produce cells that expressed a high level of DR $\alpha\beta$ 21. 1 x 10⁵ cells/well of a 96-well plate were irradiated (15,000 Rad), and wild-type or mutant SE, was added. After a brief incubation period (45 min, 37°C), unbound SE was rinsed from the culture plates using warm media. The cells were cultured in RPMI-1640 (USAMRIID) with 5% FBS for 72 h, and pulsed-labelled for 12 h with 1 μ Ci [³H]-thymidine (Amersham,

Arlington Heights, IL). Cells were harvested onto glass fiber filters, and [³H]-thymidine incorporation into the cellular DNA was measured by a liquid scintillation counter (BetaPlate, Wallac Inc., Gaithersburg, MD). Splenic mononuclear cells or human peripheral blood mononuclear cells were obtained by buoyant density centrifugation (Histopaque; Sigma Chemical Comp.) and washed three times. The cells were resuspended in medium containing 5% fetal bovine serum (FBS), and 100 µl (4 x 10⁵ cells) of the cell suspension was added to triplicate wells of 96-well flat bottom plates. The mononuclear cells were cultured (37°C, 5% CO₂) with WT or mutant SEA. After 3 days the cultures were pulsed (12h) with 1 µCi/well of [³H]thymidine (Amersham, Arlington Heights, IL) and incorporated radioactivity was measured by liquid scintillation.

Gel electrophoresis and immunoblotting analysis.

The protein preparations were analyzed by SDS-PAGE (12%) and stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Comp. St Louis, MO) in methanol (10% v/v) acetic acid (10% v/v). The proteins separated by SDS-PAGE (not stained) were transferred to nitrocellulose membranes (Bio-Rad Lab. Inc., Melville, NY) by electroblotting, and the membranes were then blocked (12 h, 4°C) with 0.2% casein in a buffer consisting of 50 mM sodium phosphate, 140 mM sodium chloride, pH 7.4 (PBS). The membrane was then incubated (1 h, 37°C, shaking) with 2 µg/mL of affinity-purified anti-toxin antibody (Toxin Technology, Sarasota, FL) in PBS with 0.02% casein. After the membranes were thoroughly washed, peroxidase-conjugated goat anti-rabbit IgG (Cappel/Organon Teknika Corp., West Chester, PA) was added (1:5,000) and the membranes were incubated for 1

h (37°C) with shaking. The unbound antibody was removed by washing with PBS and bound antibody was visualized by using a Bio-Rad peroxidase development kit (Biorad, Hercules, CA). For quantitation, dilutions of wild-type preparations were immobilized on nitrocellulose membranes by using a Slot-Blot apparatus (Bio-Rad). The membrane was removed from the Slot-Blot apparatus and unreacted sites were blocked (12h, 4°C) with 0.2% casein in PBS. After washing once with the PBS, the membrane was incubated (1h, 37°C) with 2 µg/mL rabbit affinity purified anti-toxin antibody (Toxin Technology) in PBS that contained 0.02% casein. After four washes, the bound rabbit antibody was reacted with goat anti-rabbit IgG conjugated with horseradish peroxidase (1 h, 37°C) and the blots were developed using enhanced chemiluminescence (ECL; Amersham Life Sciences, Arlington Heights, IL) or similar methods. The amount of mutant protein was measured by densitometry (NIH Image 1.57 software, National Institutes of Health, Bethesda, MD) of exposed X-ray film. Standard curves were prepared by plotting the mean of duplicate densitometric readings for each dilution of toxin standard. The resulting values were fitted to a straight line by linear regression. Concentrations of proteins were determined by comparing mean values of various dilutions of the mutant to the standard curve.

Biological activities and Immunizations.

Male C57BL/6 mice, 10 to 12-weeks old, were obtained from Harlan Sprague-Dawley, Inc. (Frederick Cancer Research and Development Center, Frederick, MD). The lethal effect of WT or mutant SEA was evaluated as described in Stiles *et al.* (1993) *Infect. Immun.* **61**, 5333-5338. For immunizations, mice were given by interperitoneal (ip) injections either 2 or

10 µg of WT or mutant toxin in 100 µl of adjuvant (RIBI, Immunochem Research, Inc. Hamilton, MT or alum), or adjuvant only, and boosted (ip) at 2 and 4 weeks. Serum was collected from tail veins one week after the last immunization. Mice were challenged 2 weeks after the last injection with toxin and lipopolysaccharide (LPS, 150 µg) from *E. coli* 055:B5 serotype (Difco Laboratories, Detroit, MI). Challenge controls were adjuvant-immunized or non-immunized mice injected with both agents (100% lethality) or with either wild type toxin or LPS. No lethality was produced by these negative controls. Monkeys were immunized with the antigen in the right leg, caudal thigh muscles. Each received three intramuscular immunizations with a superantigen vaccine plus adjuvant. Control monkeys received 0.5 ml total volume of adjuvant (Alhydrogel, Michigan Department of Public Health) and sterile PBS using the same techniques and equipment as the immunized monkeys. Immunizations were administered 28±2 days apart and consisted of 20 µg of the vaccine in adjuvant in a total volume of 0.5 ml. Immunizations were administered on day 0, 28±2, and 56±2 using a 23-27 ga 1/2-5/8" needle attached to a 1 ml tuberculin syringe into the caudal thigh.

Antibody assay.

Microtiter plates were coated with 1 µg/well of WT toxin in 100 µl of PBS (37°C, 2 h). After antigen coating, the wells were blocked with 250 µl of casein 0.2% in PBS for 4 h at 37°C and then washed four times with PBS containing 0.2% Tween 20. Immune or nonimmune sera were diluted in PBS containing 0.02% casein and 100 µl of each dilution was added to duplicate wells. After each well was washed four times, bound antibody was detected with horse radish

peroxidase (Sigma Chemical Comp., St. Louis, MO)
 labelled goat anti-species specific IgG (37°C, 1 h),
 using O-phenylenediamine as the chromogen. Mean of
 duplicates OD (absorbance at 490 nm) of each treatment
 5 group was obtained and these data were compared on the
 basis of the inverse of the highest serum dilution
 that produced an OD reading four times above the
 negative control wells. For negative controls,
 antigen or serum was omitted from the wells.

10 Superantigen binding and TCR subset analysis.

Cells from the mouse B-lymphoma line A20 (ATCC,
 Rockville, MD) ($2-4 \times 10^5$ cells) were incubated (40 min
 at 37°C) with WT or mutant toxin in Hanks balanced
 salt solution containing 0.5% bovine serum albumin
 15 (HBSS, USAMRIID). The cells were washed with HBSS and
 incubated with 5 µg of affinity-purified anti-toxin
 antibody in HBSS (4°C, 45 min). Unbound antibody was
 removed and the bound antibody was detected with
 fluorescein isothiocyanate (FITC)-labelled, goat anti-
 20 rabbit IgG (Organon Teknika Corp., Durham, NC).
 Unbound antibody was removed and the cells were
 analyzed by with a FACSort flow cytometer (Becton
 Dickinson & Co. , Mountain View, CA).

For TCR subset analysis, splenic mononuclear
 25 cells were obtained from mice immunized with WT or
 mutant toxin. The mononuclear cells were incubated
 (37°C) with WT toxin (100 ng/mL) for 5 days and then
 cultured in 85% RPMI-1640, 10% interleukin-2
 supplement (Advanced Biotechnologies Inc., Columbia,
 30 MD) with 5% FBS for an additional 5 days. The T cells
 were washed twice and stained with anti-TCR
 (Biosource, Camarillo, CA) or anti-Vβ specific TCR
 (Biosource, Camarillo, CA) (45 min, 4°C). All cells
 analyzed were positive for T cell marker CD3+ and
 35 expressed the CD25 activation marker (data not shown).

Controls were incubated with an isotype matched antibody of irrelevant specificity. Unreacted antibody was removed, and the cells were incubated with an FITC-labelled, anti-mouse IgG (Organon Teknika Corp, Durham, NC) on ice for 30 min. The cells were washed and analyzed by flow cytometry (FACSort).

LPS potentiation of SE toxicity in mice.

C57BL/6 or BALB/c mice weighing 18-20 g (Harlan Sprague Dawley, Inc., Frederick Cancer Research and Development Center, Frederick, MD) were each injected intraperitoneally (i.p.) with 200 μ l of PBS containing varying amounts of SEA, SEB, or SEC1, TSST-1, or SpeA followed 4 h later with 75 or 150 μ g of LPS (200 μ l/i.p.). Controls were each injected with either SE (30 mg) or LPS (150 mg). Animals were observed for 72 h after the LPS injection. Calculations of LD50 were done by Probit analysis using 95% fiducial limits (SAS Institute Inc., Cary, NC).

The biological effects of SEA and SEB were also tested in transgenic C57BL/6 mice (GenPharm International, Mountain View, CA) deficient in MHC class I or II expression [Stiles et al. (1993) *Infect. Immun.* **61**, 5333-5338], as described above, using a single dose of toxin (30 μ g/mouse). Genetic homozygosity was confirmed by Southern analysis of parental tail DNA, using β 2 microglobulin and MHC class II β DNA probes.

Detection of cytokines in serum.

Mice (n=18 per group) were injected with toxin (10 μ g), LPS (150 μ g), or toxin plus LPS. Sera were collected and pooled from three mice per group at each time point (2, 4, 6, 8, 10, 22 h) after LPS injection. Sera were collected at various time points following toxin injection (-4 h, or 4h before LPS injection, for

data tabulation). Collection of LPS control sera began at the time of injection (0 h).

Serum levels of TNF α and IL- α were detected by an enzyme linked immunosorbent assay (ELISA). TNF α was first captured by a monoclonal antibody against mouse TNF α (GIBCO-BRL, Grand Island, NY) and then incubated with rabbit anti-mouse TNF α antibody (Genzyme, Boston, MA). The ELISA plate was washed and peroxidase conjugate of anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN) added to the wells. After washing the plate and adding substrate (Kirkegaard and Perry, Gaithersburg, MD), TNF α concentrations were measured using the mean A450 reading of duplicate samples and a standard curve generated from recombinant mouse TNF α (GIBCO-BRL). Serum levels of IL-1 α were determined from the mean reading of duplicate samples with an ELISA kit that specifically detects murine IL-1 α (Genzyme, Boston, MA). The standard error of the mean (SEM) for TNF α and IL-1 α readings was +/- 5%.

Quantitation of IL-6 and IFN γ were measured by bioassays [See *et al.* (1990) *Infect. Immun.* **58**: 2392-2396]. An IL-6 dependent cell line, 7TD1 (kindly provided by T. Krakauer), was used in a proliferative assay with serial two-fold dilutions of serum samples assayed in triplicate. Proliferation of 7TD1 cells in a microtiter plate was measured by uptake of [3 H]-thymidine (1 μ Ci/well; Amersham, Arlington Heights, IL) and the activity of IL-6 from serum was compared to a recombinant mouse IL-6 standard (R and D Systems, Minneapolis, MN) as previously described [See *et al.* (1990) *Infect. Immun.* **58**: 2392-2396]. The SEM of triplicate samples was +/- 10%.

IFN γ was measured by the reduction of vesicular stomatitis virus (New Jersey strain) cytopathic

effects on L929 cells, as previously described [Torre
et al. (1993) *J. Infect. Dis.* **167**, 762-765]. Briefly,
 serial two-fold dilutions of serum were made in
 duplicate and added to microtiter wells containing
 5 L929 cells (5×10^4 /well). After incubating 24 h,
 virus (5×10^5 PFU/well) was added and the cytopathic
 effects measured at 48 h by absorbance readings (570
 nm) of reduced 3-[4, 5-dimethylthiazol-2-yl]-2,5
 diphenyl tetrazolium bromide (Sigma). The activity of
 10 each serum sample was determined using recombinant
 mouse IFN γ as a standard (Biosource, Camarillo, CA).
 The SEM of duplicate samples was \pm 5%.

Protein production.

Reagents and Solutions:

15 Bacterial Wash Buffer #1: 10mM Tris/30mM NaCl, 10ml of
 1M Tris (Sigma), pH 7.6, 6ml of 5M NaCl (Sigma),
 adjust volume with H₂O to 1 Liter.
 Inclusion Product Wash Buffer #2: 10mM Tris/100mM
 NaCl, 1 ml of 1M Tris, pH 8.0, 2 ml of 5M NaCl, adjust
 20 volume with H₂O to 100 ml
 Lysis Buffer: 375 μ L of 1M Tris, pH 8.0, 30 μ L of 500mM
 EDTA (Sigma), 300 μ L of 5M NaCl, 15 mL final vol.
 Refolding Buffer: 4.8g of Urea (4M Urea final soln.;
 Sigma), 2ml of 1M Tris, pH 8.5, 100 μ L of 1M DTT (final
 25 conc. of 5 mM; Life Technologies).
 DNase: 100 U/ μ L, frozen aliquots, (reconstituted with
 Lysis Buffer; Pharmacia Biotech). Lysozyme: 10 mg/ml
 stock, frozen aliquots; (reconstituted with Lysis
 Buffer; Sigma). DOC: (Sodium Deoxycholate); powder
 30 (Sigma). DTT: (Dithiothreitol); 1 M in H₂O, frozen
 aliquots (Life technologies). IPTG: (isopropyl β -D-
 thiogalactopyranoside; 500 mM stock; Life
 technologies). Kanamycin: (50 mg/ml stock in H₂O;
 Sigma)

A single bacterial colony from a fresh streak plate of BL21 (DE3) harboring the expression plasmid was used to inoculate a starter culture of 100 ml of media (e.g. LB or Terrific Broth), containing the appropriate antibiotic (kanamycin, 50µg/ml final or 75 µg/mL ampicillin). The culture was grown for 12-16 hours (overnight) in an incubator/shaker at 37°C. A shaker incubator with chiller/heater combination was used to provide reliable temperature control.

Preparative cultures were inoculated with 10-50 ml of the fresh seed culture per 1 L of pre-warmed (37°C) media, containing antibiotic (e.g. 50 µg/ml kanamycin). Cultures (37°C) were grown and the bacterial density was monitored in 30 min intervals beginning 2 hours after inoculation. Incubator temperature was then dropped to induction temperature of 30°C. A final concentration of 1 mM IPTG was added when culture reached 1/2 log growth incubation was continued (30°C) for an additional 2-4 hours. The bacterial cultures were transferred to 500ml Sorvall centrifuge bottles and bacteria pelleted by centrifugation in a Sorvall RC5C centrifuge (5000 rpm, 20 min, 4°C, GS3 rotor). The supernatant was discarded and pellets were held on ice (4°C). The bacterial pellets were resuspended in 400ml of Bacterial Wash Buffer #1. Pellet the bacteria by centrifugation in Sorvall RC5C centrifuge (5000 rpm, 20 min, 4°C, GS3 rotor). Supernatant was discarded the bacterial pellet resuspended in Bacterial Wash Buffer #1; 50 ml of buffer/2.5 ml of pelleted bacteria. The bacterial pellet was concentrated by centrifugation and frozen (-20°C). Bacterial pellet was rapidly thawed in a 37°C water bath, resuspended by mixing (1-2 min) pellet in 15ml Lysis Buffer. Next

400 μ L lysozyme (10mg/ml stock) was added and mixed for 30 min (20-22°C) on rotator.

Dry DOC (20 mg) was stirred into bacterial suspension with a clean pipette for 10 min in a 37°C water bath and 500 Units of DNase was then added. After mixing (20-22°C) for 30 min, the lysed bacteria were transferred by pipet to a clean 50 ml high speed centrifuge tube and the inclusion granules were pelleted by centrifugation (Heraeus Sepatech rotor, Baxter Biofuge 17R table-top centrifuge, 11000 rpm, 15 min, 4°C).

The inclusions were washed 2x by centrifugation in 5ml Inclusion Product Wash Buffer #2 (Heraeus Sepatech rotor, Baxter Biofuge 17R table-top centrifuge, 11000 rpm, 15 min, 4°C), resuspended in 20ml Refolding Buffer and rotated 2 hour (20-22°C). The solution was cleared by centrifugation and nondissolved protein removed. Supernatants were dialyzed against 2L of Phosphate Buffered Saline (PBS, pH 7.4), for 12-16 hours (4°C) and any precipitated material removed by centrifugation. The cleared, PBS-equilibrated product was filter sterilized (0.45 micron filter) and frozen until use (-20°C).

Western Immunoblots. Proteins (approx. 2 ug/lane) were electrophoresed through 12% polyacrylamide gels in the presence of SDS (1%), with dithiothreitol (2 mM). Gels were then electroblotted onto a protein-binding membrane (Amersham), and blocked (2 h, 37°C) with 0.2% casein in PBS. The membrane was then incubated (1 h, 37°C) with a 1/200 dilution of affinity-purified, rabbit anti-SpeA or SpeB (Toxin Technologies, Sarasota, FL). Unbound antibody was washed from the membrane using PBS, and bound antibody was detected with peroxidase conjugated, goat anti-

rabbit antisera, using a commercial color development kit (BioRad, Richmond, CA).

EXAMPLE 1

Molecular modelling and structural studies of
 5 staphylococcal and streptococcal superantigens:
bacterial superantigens share common 3-dimensional
structure.

Comparison of amino acid sequences (**Fig. 1**) suggested that bacterial superantigens fall into
 10 groups consisting of (1) SEA, SED and SEE, (2) SEB, staphylococcal enterotoxins C1-C3 (SEC1-3), the streptococcal pyrogenic exotoxins A (SPE-A) and C (SPE-C), (3) TSST-1 and (4) the exfoliative toxins (ETA, ETB) and streptococcal pyrogenic exotoxin B
 15 (SPE-B), which are the most distant from the others in sequence. Although not available to the inventor when the inventions were first conceived and proof of principle was obtained, the x-ray crystallographic structures of several bacterial superantigens are now
 20 known. Diverse superantigens, such as SEB and TSST-1, appear to have little sequence in common, yet they exhibit homologous protein folds composed largely of β strands [Prasad, G.S. et al. (1993) *Biochemistry* **32**, 13761-13766; Acharya, R.K. et al. (1994) *Nature* **367**,
 25 94-97; Swaminathan, S. et al. (1992) *Nature* **359**, 801-806] within two distinct domains. Differences between the proteins are located primarily in highly variable regions comprised of several surface loops, such as the disulfide-bonded loop which is absent from TSST-1
 30 and at the amino terminus.

The X-ray crystal structures of SEB and TSST-1 complexed with HLA DR1 are known [Kim, J. et al. (1994) *Science* 266, 1870-1874 ; Jardetzky, T.S. et al. (1994) *Nature* 368, 711-718] and this data was useful

to fully explain our results concerning attenuation of the superantigens by site-specific mutagenesis. The region of HLA DR1 that contacts SEB consists exclusively of α subunit surfaces. The main regions of SEB involved are two conserved sites: a polar pocket derived from three β strands of the β barrel domain and a highly solvent-exposed hydrophobic reverse turn. The polar binding pocket of SEB contains a glutamate and two tyrosines that accommodate Lys39 of the α subunit of HLA DR1, while the hydrophobic region consists of a leucine and flanking residues that make several contacts with the HLA DR α chain. The HLA DR1 binding sites of both TSST-1 and SEB overlap significantly. The hydrophobic binding contacts of other SAg with the HLA DR α chain have been proposed [Ulrich et al. (1995) *Nature, Struct. Biol.* **2**, 554-560] to be similar to those found in SEB and TSST-1. A motif consisting of a leucine in a reverse turn [Ulrich et al. (1995), *supra*] is conserved among bacterial superantigens and may provide the key determinant (hydrophobic or otherwise) for binding HLA-DR. However, TSST-1 does not have a highly charged residue in the polar pocket that interacts with Lys39 of the HLA DR α chain and uses an alternative conformational binding mode that allows TSST-1 to interact with HLA DR1 β -chain residues and the carboxy-terminal region of the antigenic peptide.

Both SEA and SEE bind to the β subunit of DR by means of a single zinc atom [Fraser, J.D. et al. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5507-5511]. The amino-terminal domain of SEA interfaces with the HLA DR α chain [Ulrich et al. (1995), *supra*], while SEA C-terminal domain residues His187, His225 and Asp227 form a zinc-coordination complex, likely with His-81 from the β chain of an adjoining HLA DR molecule.

However, our results have shown that binding of
 superantigen to the HLA DR β subunit does not directly
 stimulate T cells [Ulrich et al. (1995), supra] but
 increases the potential of the bound SEA to interact
 5 with the α chain of another HLA DR, thus increasing
 the biological potency.

EXAMPLE 2

Molecular modelling and structural studies of
 10 staphylococcal and streptococcal superantigens: A
detailed protein structure analysis of SEB and SEA
suggested that all bacterial superantigens have a
common mechanism for binding MHC class II receptors.

A least-squares superimposition of the unbound
 15 molecules of modeled SEA and the crystal structure of
 SEB, aligned according to their structurally conserved
 α -helical and β -strand regions, exhibited a global
 folding pattern which is very similar. Differences
 between the two structures are calculated to be
 20 located primarily in loops of low sequence homologies,
 with the largest positional deviations occurring
 between structurally conserved regions of residues 18-
 20, 30-32, 173-181, 191-194, and the cysteine-loop
 region (90-111). Only one of these regions in SEB
 25 makes significant contact (residue Y94 in particular)
 with the HLA-DR1 molecule [Jardetzky, T.S. et al.
 (1994) *Nature* **368**, 711-718].

The binding interface between SEB and HLA-DR1
 consists principally of two structurally conserved
 30 surfaces located in the N-terminal domain: a polar
 binding pocket derived from three β -strand elements of
 the β -barrel domain and a hydrophobic reverse turn.
 The binding pocket of SEB contains residues E67, Y89
 and Y115, and binds K39 of the DR α subunit. For SEA,

the binding interface with the DR molecule is modeled to contain a similar binding pocket consisting of residues D70, Y92 and Y108. Mutation of residue Y89 in SEB or Y92 in SEA to alanine (**Fig. 2**) resulted in 100-fold reduction in DR1 binding. The substitution of alanine for Y89 in SEB and Y92 in SEA eliminates the hydrogen bond with K39 and disrupts packing interactions with adjacent protein residues. Modeling of the SEA mutant Y92A predicts an increase in solvent-accessible surface area for Y108 by a factor of two greater than the wild-type structure, allowing the formation of a hydrogen bond to the carboxylate group of D70 and thus disrupting key anchoring and recognition points for HLA-DR1. This effect is expected to be somewhat less in SEB due to the longer side chain at E67. Substitution of SEB Y115 with alanine also resulted in 100-fold reduction of binding. In contrast, the same replacement of Y108 in SEA yielded little to no change in DR1 binding (**Fig. 2a**), suggesting the primary importance of SEA residues Y92 and D70 for stabilizing interactions with K39. The K39 side chain of DR α forms a strong ion-pair interaction with the SEB E67 carboxylate group and hydrogen bonds with the hydroxyl groups of Y89 and Y115. Substitution of SEB E67 by glutamine reduced binding affinity by 100-fold (**Fig. 2**), reflecting the replacement of the strong ionic bond with a weaker hydrogen bond. To optimize ion-pair interactions of the analogous SEA site, the shorter carboxylate side chain of D70 is predicted to shift K39 of DR α , weakening interactions with SEA Y108. The substitution of alanine for SEA Y108 is thus more easily accommodated than the homologous substitution of SEB Y115, without loss in DR1 binding.

Comparisons of the polar pocket with other bacterial superantigens were then made. SEC1-3 and SPE-A have conserved the critical DR1 binding-interface residues (**Fig. 1**), and share with SEB and SEA secondary structural elements of the DR1-binding surfaces. Asparagine in SED (N70) replaces the acidic side chain present in SEA, SEB, SPE-A and SEC1-3. Accordingly, for SED the salt bridge of the polar pocket is likely to be replaced by a hydrogen bond.

Overall DR1 affinities for SED and SEA appeared to be equivalent (**Fig. 2b**), indicating that other interactions may compensate for the absence in SED of the ion-pair found in the other superantigens. For the case of TSST-1, mutating DR α residues K39 to serine or M36 to isoleucine has been shown to greatly reduce binding [Panina-Bordignon *et al.* (1992) *J. Exp. Med.* **176**: 1779-1784]. Although primarily hydrophobic, the critical TSST-1 structural elements are conserved with the SEA and SEB polar binding pocket. SEB residues Y89 and Y115 are homologous to T69 and I85 in TSST-1, respectively, and SEB E67 is replaced by I46. These TSST-1 residues are positioned in a conserved β -barrel domain found in both SEB and SEA. However, the TSST-1 site lacks polarity equivalent to SEB/SEA, and hydrogen bonding with the hydroxyl of TSST-1 residue T69 would require that DR α K39 extend 5 Å into the pocket. TSST-1 binding utilizes an alternative strategy [Kim *et al.* (1994) *Science* **266**: 1870-1874] consisting of hydrophobic contacts centered around residue I46, and potential ionic or hydrogen bonds bridging DR α residues E71 and K67 to R34 and D27, respectively, of TSST-1.

The hydrophobic region of the binding interface between SEB and the HLA-DR1 molecule consists of SEB residues 44-47, located in a large reverse turn

connecting β -strands 1 and 2 of SEB. These residues appear to make strong electrostatic interactions with DR α through their backbone atoms. The mutation of L45 to an arginine reduced overall HLA-DR1 binding greater than 100-fold (**Fig. 2b**), attributable to the less energetically favorable insertion of a highly charged residue into a hydrophobic depression on the DR1 molecule. The modeled DR1-SEA complex presents similar interactions with the SEA backbone atoms, with the exception of a glutamine (Q49) replacing SEB Y46. Mutation of L48 to glycine in SEA (homologous to L45 of SEB) has been reported to decrease T-cell responses. SEB L45 and the comparable L30 of TSST-1 are the most extensively buried residues in the DR1 interface. The leucine is conserved among the bacterial superantigens (**Fig. 3**) and may provide the necessary hydrophobic structural element for surface complementarity with DR1, consistent with the mutagenesis data for SEB and SEA.

The inventor has performed similar structure and function studies with TSST-1, SEC1 and SPE-A.

EXAMPLE 3

Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: Some interactions of bacterial superantigens with MHC class II receptors are not conserved but are less important than the hydrophobic loop and polar pocket binding sites.

In determining the overall affinity of the superantigen for DR1, a contributory role is played by structural variations around the common binding motifs. A short, variable structured, disulfide-bonded loop is found in SEA and a homologous longer loop in SEB. The SEB residue Y94, contained within

this loop, forms hydrophobic interactions with L60 and A61 of the DR α subunit. Replacement of Y94 with alanine partially inhibits DR1 binding (**Fig. 2a,b**). An alanine is found in SEA (A97) and SEE at the position equivalent to SEB Y94, and mutating this residue in SEA to tyrosine results in disrupted instead of stabilized interactions with DR1 (**Fig. 2a**). Although the disulfide loops differ in structure between SEA and SEB, A97 apparently contributes to the DR α binding interface in a manner similar to Y94 of SEB. Because TSST-1 lacks a disulfide loop, similar contacts with DR α are replaced by interactions with β -strands of TSST-1. In a like manner, the absence of a salt bridge between the residues K39 of DR α and E67 of SED is apparently compensated for by stabilizing interactions occurring outside of the otherwise conserved dominant binding surfaces (**Fig. 2a**).

EXAMPLE 4

Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: Superantigen interactions with T-cell antigen receptors.

The amino acid residues in contact with TCR are located in regions of high sequence variability, presenting a unique surface for interaction with the TCR. Residues implicated in TCR interactions by mutagenesis of SEA and SEB reside in variable loop regions, while TSST-1 mutants that affect TCR binding are mainly located in an α helix [Acharya, R.K. et al. (1994) *Nature* **367**, 94-97; Kim, J. et al. (1994) *Science* **266**, 1870-1874]. Specifically, mutations that diminish T-cell receptor recognition of SEB include residues N23, Y61, and the homologous SEA N25 or Y64 (**Fig. 2c**). SEA residues S206 and N207 also control

T-cell responses [Hudson, et al. (1992) *J. Exp. Med.* **177**: 175-184]. Mutants of the polar binding pocket, SEA Y92A and SEB Y89A, equivalently reduced T-cell responses (**Fig. 2c**), reflecting the observed
 5 decreases in DR1-binding (**Fig. 2a, b**). While supporting reduced T-cell responses, mutants SEA Y64A and SEB Y61A retained normal affinities for DR1 (**Fig. 2a-c**).

EXAMPLE 5

10 Animal models for determining biological activity of bacterial superantigens: Mouse.

When compared to primates, mice are not very susceptible to the toxic effects of SE, and we therefore sought to increase sensitivity with a
 15 potentiating dose of lipopolysaccharide (LPS) from Gram-negative bacteria [Stiles et al. (1993) *Infect. Immun.* **61**, 5333-5338]. There was no apparent effect in control animals injected with any of the SE (up to 30 µg/mouse) or LPS (150 µg/mouse) alone (Table 1).
 20 Incremental injections of LPS were also not lethal, when given in amounts up to 250 µg/mouse (data not shown). However, mice died between 24-48 h after SE and LPS were given to the same animal (Table 1). SEA was much more toxic than either SEB or SEC1 and the
 25 calculated LD50 (µg toxin/kg) of SEA, SEB, and SEC1 with 95% fiducial limits was 18.5 (6.5, 38.5), 789.0 (582.5, 1044.5), and 369.0 (197.5, 676.0), respectively.

TABLE 1. Titration of SEA, SEB, and SEC₁ in the C57BL/6 mouse lethality assay

Stimulus ^a	% Lethality (no. of mice tested) with the following dose of SE, in micrograms/mouse ^b :			
	30	10	1	0.1
SEA + LPS	93 (15) ^b	85 (20)	80 (15)	20 (10)
SEB + LPS	80 (15)	27 (15)	0 (15)	0 (15)
SEC ₁ + LPS	80 (10)	60 (10)	10 (10)	0 (10)

^aLPS was injected into each mouse (150ug) 4 h after the SE injection. Control mice injected with 150 ug of LPS (n=20) or 30 ug of SEA, SEB, or SEC₁ (n=10) survived.

^bResults are from a combination of separate experiments with five mice per experiment.

The role of MHC class I and class II molecules in SE toxicity, potentiated by LPS, was addressed by using transgenic, MHC-deficient mice (Table 2). Class II-deficient animals were unaffected by a dose of SE (30 µg) plus LPS (150 µg) that was lethal for 93% of wild-type and 30% of class I-deficient mice.

Mononuclear cells from class II-deficient animals were not able to present SEA, as measured by proliferative responses. MHC class I-deficient cells were functional in supporting T-cell proliferation, but at levels <30% of the proliferative response supported by MHC-wild-type presenting cells (Table 3). Cell surface expression levels were normal, when compared to nontransgenic C57BL/6, for A^b in class I-deficient mice, and K^b/D^b in class II-deficient mice. The T-cell responses of MHC class I- or class II-deficient mice were essentially equivalent to wild-type when SEA was presented by mononuclear cells expressing both class I and II molecules (Table 3).

TABLE 2. Lethality of SEA and SEB in C57BL/6 mice lacking MHC class I or class II

Stimulus ^a	% Lethality (no. of mice tested) with the following MHC class phenotype		
	I ⁻ II ⁺	I ⁺ II ⁻	I ⁺ II ⁺
5 SEA + LPS	30 (10)	0 (5)	93 (15)
SEA + LPS	ND ^b	0 (5)	80 (15)
10 SEA only	0 (2)	0 (2)	0 (2)
SEB only	ND ^b	0 (2)	0 (2)
LPS only	0 (5)	0 (5)	0 (5)

15 ^a Mice were injected with 30 ug of SEA or SEB and, 4h later, with 150 ug of LPS, as indicated. Control mice were injected with only SEA, SEB, or LPS.

^b ND, not determined.

20 Table 3. Mouse T-cell responses to SEA are MHC class II-dependent

T-cell/APC source ³	T-cell responses ¹	
	0.1 µg/ml SEA	1 µg/ml SEA
25 Wild-type C57/BL6 mouse/autologous	430,000 cpm ²	700,000 cpm
MHC class I knock-out	117,000 cpm	167,000 cpm
30 C57/BL6 mouse/autologous		
MHC class II knock-out	8,000 cpm	33,000 cpm
35 C57/BL6 mouse/autologous		
Wild-type C57/BL6 mouse/wild-type	305,000 cpm	307,000 cpm
40 MHC class I knock-out C57/BL6 mouse/wild-type	420,000 cpm	445,000 cpm
MHC class II knock-out C57/BL6 mouse/wild-type	310,000 cpm	322,000 cpm
45		

¹Cultures of mononuclear cells derived from mouse spleens, cultured for 3 d with the indicated amount of SEA.

5 ²Data represent the mean of triplicate determinations (<10 SEM) of [³H]thymidine incorporation.

³Antigen presenting cells (APC) were isolated from spleens of the indicated mouse strain and added to cultures.

10 The serum levels of TNF α , IL-1 α , IL-6, and IFN γ in mice injected with SEA, LPS, or SEA plus LPS were measured at various times following injection (**Fig. 4**). Compared to mice injected with either SEA or LPS alone, the serum levels of TNF α , IL-6, and IFN γ had
15 increased 5-, 10-, and 15-fold, respectively, in animals given SEA plus LPS. SEA alone did not elicit any detectable increase of TNF α , IL-6, or IFN γ above background. In contrast to the other cytokines, IL-1 α levels in mice injected with SEA plus LPS resulted in
20 a simple additive effect.

 Serum levels of TNF α , IL-6, and IFN γ were maximal 2-4 h after the LPS injection, but returned to normal by 10 h. The concentration of IL-1 α in mice given SEA plus LPS had also peaked 2 h after the LPS injection,
25 but stayed above background for the remaining determinations. Levels of IL-1 α in mice given only LPS or SEA peaked at 4 and 6 h, respectively. Unlike profiles for other cytokines, the highest amount of IL-1 α in mice injected with SEA and LPS corresponded
30 to the peak stimulated by SEA, but not LPS.

 This animal model was used in various stages of developing the inventions, as a means of assessing the physiological activity of mutated superantigens. Control animals survived the maximum dose of either SE
35 or LPS, while mice receiving both agents died. Wild-type SEA was 43-fold more potent than SEB and 20-fold more potent than SEC1. By using BALB/c mice the

toxicity of SEB was 10-20 fold higher. These data confirmed that the toxicity of SE was mainly exerted through a mechanism dependent on expression of MHC class II molecules and was linked to stimulated cytokine release. Thus this was a relevant preclinical model that could be used to predict human responses.

EXAMPLE 6

Animal models for determining biological activity of bacterial superantigens: Rhesus monkey

The physiological responses of the rhesus monkey to bacterial superantigens is probably identical to humans, with the exception of sensitivity [Bavari and Ulrich (1995) *Clin. Immunol. Immunopath.* **76**:248]. Generally SEB intoxicated monkeys developed gastrointestinal signs within 24 hours post-exposure. Clinical signs were mastication, anorexia, emesis and diarrhea. Following mild, brief, self-limiting gastrointestinal signs, monkeys had a variable period of up to 40 hours of clinical improvement. At approximately 48 hours post-exposure, intoxicated monkeys generally had an abrupt onset of rapidly progressive lethargy, dyspnea, and facial pallor. If given a lethal dose, death occurs within four hours of onset of symptoms. Only SEB has been used in challenges of rhesus monkeys to determine physiological/pathological effects. Human responses to bacterial superantigens are characterized by a rapid drop in blood pressure, elevated temperature, and multiple organ failure-the classical toxic shock syndrome (TSS). However, the respiratory route of exposure may involve some unique mechanisms. The profound hypotension characteristic of TSS is not observed, and respiratory involvement is rapid, unlike

TSS. Fever, prominent after aerosol exposure, is generally not observed in cases of SEB ingestion.

EXAMPLE 7

Targeting receptor interactions to develop vaccines.

The SEA mutants Y92A, with reduced DR1 binding, and Y64A, with reduced TCR interactions, and K14E with wild-type (control) activity were used to determine the correct receptor to target for vaccine development. The binding of WT or mutant SEA was evaluated with the MHC class II expressing murine B-cell lymphoma cell line A20 (Table 4). The binding affinity of WT SEA to mouse MHC class II (H-2^d) molecules was lower than that observed with human MHC class II expressing cells, reflecting the reduced toxicity that bacterial SAGs exert in mice. WT SEA, Y64A and K14E all had the same relative affinity to mouse MHC class II molecules. Similar to the results obtained with human MHC class II molecules, the Y92A mutant exhibited substantially reduced binding to A20 cells (Table 4).

Table 4. Biological activity of superantigen vaccines

25	toxin	T-cell anergy ¹	MHC classII binding ²	T-cell response
	SEA wild type	++++	+++	+++
30	TCR attenuated Y64A	+	+++	+/-
	MHC attenuated Y92A	-	+/-	+/-
35	Control K14E	++++	+++	+++

40 ¹Based on attenuation of T-cell response to wild-type SEA in mice immunized with the mutant or wild-type SEA.

²Binding to the mouse MHC class II+ A20 cells, measured by flow cytometry

- 5 The effect of WT SEA or site-specific SEA mutants on splenic mononuclear cells obtained from nonimmunized C57BL/6 (H-2^b) mice is summarized in Table 4. Both WT SEA and the control mutant K14E were potent T cell activators, effective at minimal
- 10 concentrations of 10 to 100 pg/mL. However, T-cell responses to Y92A were reduced at least 100-fold, compared to SEA wild type, while Y64A-stimulated responses were slightly higher than Y92A. These results confirmed that attenuation of superantigen
- 15 binding to either MHC class II or TCR molecules resulted in dramatically reduced mouse T-cell proliferation. These results may indicate that the altered toxin may compete with wild type toxin for TCR binding.
- 20 SEA WT (10 LD₅₀), site-specific SEA mutants (10 µg/mouse each) or LPS (150 µg/mice) injected alone were nonlethal to mice (Table 5). However, combining LPS with either WT SEA or mutant K14E resulted in 100% lethality. For those mice receiving both LPS and WT
- 25 or K14E SEA, 80% were dead by 24 h and 100% by 48 h. In contrast, 100% of Y92A and 80% of Y64A injected mice (coadministered with LPS) survived. The average time to death for the 20% of mice that did not survive Y64A injection occurred at 48 to 72 h. These
- 30 *in vivo* data correlated well with the results obtained with the lymphocyte cultures. It was concluded that the observed attenuation of toxicity in mice was a direct result of the reduced T-cell proliferation.

Table 5. Biologic effect of wild type (WT)
staphylococcal enterotoxin A (SEA) and SEA mutants.

5	Protein	No. live/total
	WT	0/10
	K14E	0/10
	Y64A	8/10
	Y92A	10/10
10		

NOTE. Mice were given 10 LD₅₀ (10ug) of WT or mutant SEA. Lipopolysaccharide (150 ug/mouse) was injected 3 h later.

Having established that attenuation of receptor
 15 binding resulted in reduced toxicity, we next examined
 the immunogenicity of the SEA mutants. Mice were
 immunized with WT or mutant SEA. Control mice
 received adjuvant only or were left untreated. One
 week before challenge with WT SEA, mice were bled and
 20 serum antibody titers were determined for each group
 (Table 6). Mice immunized with the 2 µg of Y64A or
 Y92A had serum antibody titers of 1:5000 and 1:1000,
 respectively. Immunization with 2 µg of WT SEA or
 control mutant resulted in titers of 1:5,000 and
 25 1:10,000, respectively. The highest immunizing dose
 (10 µg/mouse) was most effective for all animals,
 resulting in antibody titers which were greater than
 1:10,000. All mice were challenged with 10 LD₅₀ of WT
 SEA (potentiated with LPS). The survival data
 30 correlated well with the levels of serum antibodies in
 immunized mice. All mice that were vaccinated with 10
 µg of Y64A or Y92A, survived the lethal challenge dose
 of WT SEA. Slightly less protection was afforded by
 the lower vaccination dose of mutant Y64A or Y92A.
 35 All mice immunized with both doses of WT SEA survived

the lethal challenge with WT potentiated with LPS. Mice immunized with mutant K14E exhibited survivals of 100% and 80% for high and low vaccination doses, respectively. All nonimmunized or control mice that
 5 were vaccinated with adjuvant alone died when challenged with WT SEA and a potentiating dose of LPS.

10 Table 6. Mice immunized with attenuated forms of staphylococcal enterotoxin A (SEA) produce high titers of neutralizing antibody.

	Immunizing agent	Dose (ug/mouse)	Anti-SEA antibody titer*	No. live/total
15	WT	2	10,000-50,000	10/10
		10	10,000-50,000	10/10
	K14E	2	5,000-10,000	8/10
		10	10,000-50,000	10/10
	Y64A	2	5,000-10,000	6/10
20		10	10,000-50,000	10/10
	Y92A	2	1,000-5,000	2/10
		10	10,000-50,000	10/10
	Adjuvant		50-100	0/10

25 NOTE. Mice were given 10 LD₅₀ of wild type (WT) SEA challenge followed by potentiating dose of lipopolysaccharide (150 ug/mouse) 3 h later.

*Reciprocal of serum dilution resulting in optical density reading four times above negative controls (wells
 30 containing either no SEA or no primary antibody).

EXAMPLE 8

Immune recognition of SAg mutants.

Bacterial SAGs induce clonal anergy of specific
 35 subsets of T cells in mice. It was possible that the loss of sensitivity to WT SEA among the mice vaccinated with the attenuated mutant forms

represented a state of specific non-responsiveness instead of specific immunity. To address this issue, lymphocyte responses to SEA WT were measured with splenic mononuclear cells collected 2 weeks after the
5 third immunization. As expected, lymphocytes from mice that were immunized with WT SEA or control SEA mutant showed little to no proliferation when incubated with the WT SAg. In contrast, lymphocytes obtained from control mice or those immunized with
10 either Y64A or Y92A all responded vigorously to the WT SEA (**Fig. 5**). The TCRs used by T cells from the SEA-vaccinated mice were then characterized by flow cytometry. T cells from immunized or control mice were incubated with WT SEA in culture for 7 days,
15 followed by a 5 day expansion in IL-2 containing medium. Distinct populations of activated TCR V β 11 positive cells were observed with T cells from mice immunized with Y92A and Y64A, representing 48% and 40% of T cells, respectively. However, V β 11 expressing
20 cells obtained from SEA WT or K14E immunized mice were about 1% and 6% of the total T-cell population, respectively, suggesting that this subset was nonresponsive to restimulation with the WT SAg. T cells bearing V β 17a, 3, 7, and 10b were unchanged for
25 all mice. It was apparent that T-cell responses to both the TCR and MHC class II binding-attenuated SEA mutants were similar to each other, but differed from responses to control or WT molecules. These results suggested that an alternative, perhaps conventional
30 antigen processing mechanism was functioning in presentation of the SAg mutants Y64A and Y92A.

EXAMPLE 9Rhesus monkey immunizations with monovalent vaccines.

5 The SEA vaccine L48R, Y89A, D70R (A489270) and
SEB vaccine Y89A, Y94A, L45R (B899445) were used to
immunize rhesus monkeys. The animals received a total
of three i.m. injections (10-20 µg/animal), given at
monthly intervals. Rhesus monkeys that were injected
10 with these vaccines had no detectable increase of
serum cytokines and no apparent toxicity. The
serological response of animals vaccinated with three
doses of formalin-treated SEB toxoid (100
µg/injection) gave results comparable to one or two
15 injections with B899445 (Table 7), suggesting that the
recombinant vaccines were very immunogenic. Immunized
rhesus monkeys survived a lethal challenge with >10
LD50 of wild-type SEB (Table 7, 8). Collectively,
these results suggest that the engineered SEB vaccine
20 is safe, highly antigenic and effective at protecting
the immunized individual from lethal aerosol exposure
to SEB.

Serum from monkeys that were immunized with the
genetically attenuated vaccine inhibited T-lymphocyte
25 responses to wild type SEB (Table 7) similarly or
better than monkeys that received the SEB toxoid.
Collectively, these results suggest that the
recombinant SAg vaccines are safe, highly antigenic,
and induce protective immunity.

30

35

Table 7. Rhesus monkey antibody responses to vaccine B899445; One injection of B899445 outperforms three injections of SEB toxoid

	Vaccine ¹ /animal #	Antibody response ²	%Inhibition of T-cell response ³	Survival SEB >20 x LD50 challenge ⁴
5				
10	preimmune sera /pooled	0.161	5	dead
	toxoid/1	0.839	0	dead
15	toxoid/2	0.893	34	live
	toxoid/3	1.308	57	live
	toxoid/4	1.447	55	live
20	B899445/1	1.788	69	live
	B899445/2	0.78	49	live

25 ¹Rhesus monkeys were immunized with one dose (20 µg injection) of B899445 vaccine or three doses of formalin-treated SEB toxoid (100 µg/injection) one month apart; both used Alum adjuvants.

30 ²Sera were collected one month after the final injection. Antibody responses were determined by ELISA and the results are shown as mean optical densities of triplicate wells (± SEM).

35 ³Rhesus monkey T cells, obtained from an untreated animal, were preincubated with diluted (1:70) serum from immunized monkeys and then cultured with wild type SEB. Data are shown as % of T cell responses, where serum of rhesus monkey injected with adjuvant only represented the 100% of response to wild type SEB.

40 ⁴Rhesus monkeys were challenged by aerosol exposure and monitored for four days.

45

50

Table 8. Engineered staphylococcal enterotoxin B vaccine efficacy in rhesus monkeys

5	Treatment ¹	Antibody titer ²	Immune protection ³
	Vaccine with adjuvant	>10,000	100%
10	Adjuvant only	<50	0%

15 ¹Rhesus monkeys (n=10) were injected i.m. with 10 µg of SEB vaccine with Alhydrogel adjuvant. A total of 3 immunizations, 1 month apart were given. Controls (n=2) received only Alhydrogel.

20 ²Serum dilution resulting in optical density readings of four times above the negative control, consisting of no SEB or serum added to the wells.

³Immunized and control rhesus monkeys were challenged with >10 LD50 of wild-type staphylococcal enterotoxin B as an aerosol.

25 Serum from B899445 immunized rhesus monkeys blocked human lymphocyte responses to wild-type superantigen when tested in ex vivo cultures (Table 7). These data again showed that the second and third injections of vaccine were approximately equivalent in stimulating neutralizing antibody responses. Normal T-cell responses to several superantigens, including the wild-type protein, were observed in immunized animals, indicating that no specific or generalized anergy occurred (**Fig. 6**).

35 **EXAMPLE 10**

A. Multivalent superantigen vaccines: Rhesus monkey immunizations.

40 Rhesus monkeys were immunized with a combined vaccine consisting of B899445 and A489270. Following the third injection, antibody recognition of wild-type bacterial superantigens was examined (**Fig.7**). High

titers of anti-SEB, SEC1 and SEA antibodies were evident.

B. Mouse immunizations.

Mice (BALB/c) were immunized with a combined vaccine consisting of SEA, SEB, SEC1 and TSST-1 (all wild-type). The antibody responses against each individual superantigen were assessed (Table 9). Antibodies were induced against each of the component antigens, providing sufficient levels to protect the mice from a lethal challenge of superantigen, potentiated with LPS. Although not shown in the Table, antibody responses against SPE-A were also observed. Mice were also immunized with individual superantigens and antibody responses against other superantigens were measured (Table 10). Each individual immunogen induced partial or complete protective antibody responses against all other superantigens tested.

TABLE 9. Superantigen cross-reactivity of antibodies from mice immunized with individual bacterial superantigens

	Immunizing ¹	Challenging ²	ELISA ³	Neutralizing ⁴
25	Toxin	Toxin	Titer	Antibody
	SEA	SEA	>1/25,000	100%
	SEA	SEB	>1/25,000	100%
	SEA	SEC1	>1/25,000	100%
30	SEA	TSST1	>1/10,000	100%
	SEB	SEB	>1/25,000	100%
	SEB	SEA	>1/10,000	100%
	SEB	SEC1	>1/2,500	100%
	SEB	TSST1	>1/10,000	100%

Table 9 Continued

	Immunizing ¹ Toxin	Challenging ² Toxin	ELISA ³ Titer	Neutralizing ⁴ Antibody
5	SEC1	SEC1	>1/10,000	100%
	SEC1	SEA	>1/10,000	100%
	SEC1	SEB	>1/25,000	100%
	SEC1	TSST1	>1/10,000	100%
10	TSST1	TSST1	<1/10,000	100%
	TSST1	SEA	<1/1,000	50%
	TSST1	SEB	<1/1,000	40%
	TSST1	SEC1	<1/1,000	40%

15 ¹Three injections with 20 ug of antigen (BALB/c mice).
 ²LPS-potentiated challenge with 10 LD₅₀s of superantigen.
 ³ELISA antibody response against an individual
 superantigen.

⁴Percent mice surviving an LPS-potentiated challenge
 20 (n=10).

Table 10. Multivalent superantigen vaccine. Mouse
immune responses.

	Immunizing toxin ¹	Challenging toxin ²	Antibody Titer ³	% survival
25	SE-A, B, C1, TSST-1	all	N/A	100%
30	" "	SEA	>25,000	100%
	" "	SEB	>25,000	100%
35	" "	SEC1	>25,000	100%
	" "	TSST-1	>6,400	100%

40

¹Total of three injections, two weeks apart, in RIBI adjuvant.

²>10 X LD₅₀, potentiated with *E. coli* lipopolysaccharide.

³Measured by ELISA.

5

EXAMPLE 11

Design of altered TSST-1 toxin vaccine, TST30.

A comprehensive study of the relationships of TSST-1 protein structure to receptor binding were undertaken to provide insight into the design of the vaccine TST30. We have discovered that TSST-1 interactions with the human MHC class II receptor, HLA-DR, are relatively weak and can be disrupted by altering only a single critical amino acid residue of the toxin. Site-directed mutagenesis of a gene encoding the toxin and expression of the new protein product in *E. coli* were then used to test the design of the vaccine. The TSST-1 gene used was contained within a fragment of DNA isolated by BglI restriction enzyme digestion of the gene isolated from a toxigenic strain of *Staphylococcus aureus* (AB259; Kreiswirth and Novick (1987) *Mol. Gen. Genet.* **208**, 84-87). The sequence of this gene is identical to all currently known TSST-1 isolates of human origin. The wild-type TSST-1 gene can be readily cloned from a number of clinical *S. aureus* isolates. The DNA fragment containing the TSST-1 gene was isolated by agarose gel electrophoresis and ligated into the prokaryotic expression vector pSE380 (Invitrogen Corp.). The DNA clone consisted of sequences encoding the leader peptide and the full length of the mature TSST-1 protein. The TST30 vaccine consists of the following mutation introduced into the toxin molecule: leucine at amino acid residue 30 changed to arginine. Two other mutations, namely Asp27 to Ala and Ile46 to Ala have also been designed. The final vaccine may incorporate one or both of these additional mutations.

The binding interface between TSST-1 and HLA-DR consists of a large relatively flat surface located in the N-terminal domain. Leucine 30 protrudes from a reverse turn on the surface of TSST-1 and forms the major hydrophobic contact with the HLA-DR receptor molecule. Mutation of the single residue leucine 30 in TSST-1 to the charged amino acid side chain of arginine is predicted to disrupt this major contact with the receptor molecule, resulting in a significant reduction in DR1 binding. This mutant molecule should therefore have lost the toxin attributes of the wild-type molecule.

TST30 was expressed as a recombinant protein in *E.coli*, as either a periplasmically secreted protein or as a cytoplasmic product. Purification was achieved by immunoaffinity chromatography or preparative isoelectric focusing after an initial ion-exchange CM-Sepharose enrichment step. The method of purification was not critical to the performance of the vaccine. Lipopolysaccharide contaminants, resulting from expression in a Gram-negative bacterium, were readily removed (as determined by limulus assay) using a variety of standard methods. The final purified vaccine is not toxic to mice at levels equivalent to 10 LD₅₀ of the native TSST-1. No indicators of toxicity were found in surrogate assays of human T-cell stimulation.

EXAMPLE 12

Structural comparisons between SEB and TSST-1 were performed using the crystal structure (Brookhaven identity code 1tss) aligned according to common secondary structural elements (Prasad, G. S., et al., 1993, Biochem. 32, 13761-13766). Site-directed mutagenesis of a gene encoding the toxin and

expression of the new protein product in *E. coli* were then used to test the design of the vaccine.

Mutating DR α residues K39 to serine or M36 to isoleucine has been shown to greatly reduce binding of
 5 TSST-1 (Panina-Bordignon, P., et al., 1992. J. Exp. Med. 176, 1779-1784). Although primarily hydrophobic, the critical TSST-1 structural elements are conserved with the SEA and SEB polar binding pocket. SEB
 residues Y89 and Y115 are homologous to T69 and I85 in
 10 TSST-1, respectively, and SEB E67 is replaced by I46. These TSST-1 residues are positioned in a conserved β -barrel domain found in both SEB and SEA. However, the TSST-1 site lacks polarity equivalent to SEB/SEA, and hydrogen bonding with the hydroxyl of TSST-1 residue
 15 T69 would require that DR α K39 extend 5 Å into the pocket. TSST-1 binding utilizes an alternative strategy consisting of hydrophobic contacts centered around residue I46, and potential ionic or hydrogen bonds bridging DR α residues E71 and K67 to R34 and
 20 D27, respectively, of TSST-1. SEB L45 and the comparable L30 of TSST-1 are the most extensively buried residues in the DR1 interface (Jardetzky, T. et al., 1994, Nature 368, 711-718; Kim, J., et al., 1994, Science 266, 1870-1874). The leucine is conserved
 25 within the bacterial superantigen protein family and provides the necessary hydrophobic structural element for surface complementarity of TSST-1 with HLA-DR. The binding interface between TSST-1 and HLA-DR consists of a large relatively flat surface located in the N-
 30 terminal domain. Leucine 30 protrudes from a reverse turn on the surface of TSST-1 and forms the major hydrophobic contact with the HLA-DR receptor molecule. Mutation of the single residue leucine 30 in TSST-1 to the charged amino acid side chain of arginine or the
 35 neutral residue alanine disrupts this major contact

with the receptor molecule, resulting in a significant reduction in DR1 binding. Significantly, loss of a methyl group in the mutation L30A was sufficient to drastically inhibit binding to HLA-DR. Thus, TSST-1
 5 interactions with the human MHC class II receptor, HLA-DR, are relatively weak and can be disrupted by altering only a single critical amino acid residue of the toxin. By reducing binding to the MHC receptor component, mutations of L30 should result in a
 10 molecule that has lost the toxic attributes of the wild-type TSST-1.

The position of introduced mutation can vary, with residues 25-35 being preferable, residues 28-32 more preferable, and residue 30 most preferable. Human
 15 T-cell responses to the mutants L30A or L30R were greatly diminished in comparison to responses to the wild-type TSST-1, confirming this prediction. The substituted amino acid can also vary, with any replacement of L30 expected to result in diminished
 20 ability to stimulate T cells.

To increase the margin of safety for therapeutic or prophylactic use of this product, an additional mutation was introduced. Because interactions with HLA-DR were eliminated by the L30 mutation, other
 25 potential sites of molecular interaction were examined. Previous studies indicated the complexity of TSST-1 interactions with T-cell antigen receptors, an issue that has not been adequately resolved. Therefore, a mutation was introduced at residue H135
 30 (H135A), forming a TSST-1 to TSST-1 contact in the crystallographic complex with HLA-DR1. T-cell responses to the mutant L30R, H135A were equal to background proliferation, in comparison to the robust stimulation apparent from wild-type TSST-1 treatment
 35 (Figure 8). The position of introduced mutation can

vary, with residues 130-140 being preferable, residues 132-137 more preferable, and residue 135 most preferable. The substituted amino acid can also vary, with any replacement of H135 expected to result in diminished ability to stimulate T cells.

Because only minor changes have been introduced into the final protein product, maximum antigenicity is maintained. Immune recognition of the TSST-1 mutants was next examined in an LPS-dependent, murine toxicity model previously described (Stiles, B.G. et al, 1993, Infect. Immun. 61:5333). Mice (Balb/C, female, 20 grams average weight; NCI) were injected (20 ug/mouse) a total of three times with TSST-1 mutant in Alhydrogel, keeping two weeks between injections. Sera were sampled two weeks after the last vaccinations and anti-TSST-1 specific antibody was measured by ELISA, using plates coated with wild-type TSST-1. Antibody titers of >120,000 were obtained by all vaccinated mice, confirming that the mutated protein was still highly immunogenic (Figure 9). Next mice were vaccinated two times with varying doses of TSST-1 L30R followed by lethal challenge with wild-type TSST-1. A challenge dose of 1.25 µg/mouse was lethal to all non-vaccinated mice, whereas vaccination with 20 µg of L30R mutant protected 20% of the mice. A challenge with 0.63 µg wild-type TSST-1 was lethal for 80% of non-immune mice, whereas 10% of mice vaccinated with 20 µg or 30% of mice vaccinated with 5 µg of L30R succumbed. A total of four vaccinations with 10 µg/mouse of TSST-1 L30R resulted in 100% protection from a 5 x LD₅₀ challenge from wild-type toxin. Three vaccinations with 10 µg/mouse of either TSST-1 L30R or L30A resulted in 70-80% protection.

Table 11: TSST-1 Mutant L30R Vaccine Dose and Immune Protection

	Vaccine Dose ¹	Challenge Dose ²	Survival ³
5	20 µg	1.25 µg	20 %
		0.63	90
		0.31	100
10	5	1.25	0
		0.63	70
		0.31	100
15	0	1.25	0
		0.63	20
		0.31	100
		0	100

¹Vaccinations 5 and 20 µg L30R or adjuvant only per mouse on day 0 and 21.

20 ²TSST-1 wild-type i.p. dose per mouse on day 31 followed by 40 µg LPS / mouse i.p. 4 hours after TSST-1 administration.

³Percent survivors 72 hr following wild-type TSST-1 challenge; 10 mice per group.

25

Table 12: TSST-1 Vaccination Schedule and Immune Protection

	Vaccination Schedule ¹	Challenge Survival ²
30	TSST-1 L30R: 3 doses	80%
	TSST-1 L30R: 4 doses	100%
35	TSST-1 L30A: 3 doses	70%
	Adjuvant only control 3 doses	0%

40

¹Vaccinations with 10 µg L30R, L30A in adjuvant or adjuvant only per mouse 0,2,4 and 6 weeks (4 dose), 0,2 and 4 weeks (3 dose).

45 ²TSST-1 wild-type i.p. dose 5 LD₅₀ per mouse 2 weeks after last vaccination, followed by 40 µg LPS / mouse i.p. 4 hours after wild-type TSST-1 administration. Percent survivors 72 hr following TSST-1 wild-type challenge; 10 mice per group.

EXAMPLE 13Design of altered SpeA toxin vaccine, SpeA42

Streptococcal pyrogenic exotoxin A (SpeA) is
 5 produced by group A *Streptococcus pyogenes* and is
 associated with outbreaks of streptococcal toxic shock
 syndrome. SpeA is also a virulence factor for invasive
 infections. The M1inv+ subclone of M1 GAS that spread
 globally in the late 1980s and early 1990s harbors the
 10 phage T14 that encodes the superantigen streptococcal
 pyrogenic exotoxin A or SpeA (Infect. Immun. 66:5592
 (1998). A typical bacterial superantigen, the 25,700 M_r
 secreted SpeA polypeptide aids in immune escape by
 targeting the primary step in immune recognition. The
 15 cellular receptors are human major histocompatibility
 complex (MHC) class II molecules, primarily HLA-DR, and
 T-cell antigen receptors (TCRs). The normal antigen-
 specific signal transduction of T cells is disengaged by
 the superantigen, which acts as a wedge to prevent
 20 contacts of MHC-bound, antigenic peptides with specific
 combining site elements of the TCR. The magnitude of
 the T-cell response is significantly greater than
 antigen-specific activation and results in pathological
 levels of proinflammatory cytokines such as tumor
 25 necrosis factor alpha (TNF- α) and interferon- γ .

Clinical isolates of *Streptococcus pyogenes*
 harboring the SpeA gene were identified by PCR
 amplification of a sequence-specific fragment from
 bacterial DNA. Specific restriction enzyme motifs for
 30 cloning were introduced into the amplified DNA fragment
 by using the following oligonucleotide primers: 5' CTCG
 CAA GAG GTA CAT ATG CAA CAA GAC 3' (SEQ ID NO:17), sense
 primer to introduce a unique NdeI site; 5' GCA GTA GGT
 AAG CTT GCC AAA AGC 3' (SEQ ID NO:18), antisense primer

to introduce a unique Hind III site. The amplified DNA fragment was ligated into the EcoRI site of a PCR-cloning plasmid (Perfectly Blunt, Invitrogen) and the resulting plasmid was used to transform *E. coli* host strain DH5 α . The HindIII/EcoRI DNA fragment containing the full-length SpeA gene minus the signal peptide was cloned into pET24 vector for expression in *E. coli* host strain BL21. Although the mutant proteins can be produced with the leader peptide sequence present, deletion of the leader peptide appeared to produce a higher yield of protein. Proteins were purified from *E. coli* inclusions and purified by cationic/anionic-exchange chromatography using standard methods (Coffman, J.D. et al., 2001. Prot. Express. Purif. in press). The method of purification was not critical to the performance of the vaccine. Lipopolysaccharide contaminants, resulting from expression in a Gram-negative bacterium, were readily removed (as determined by limulus assay) using a variety of standard methods. Two different mutants of SpeA were designed and produced based on the principle of mutating key amino acid residues involved with binding to MHC class II receptors. The first SpeA construct consists of a single mutation at residue leucine 42, while the second construct consists of a fusion between the SpeA mutant of leucine 42 and a mutant SpeB protein.

The binding interface between SpeA and HLA-DR is predicted to consist of contacts located in the N-terminal domain that are conserved with other bacterial superantigens. Leucine 42 of SpeA is predicted to protrude from a reverse turn on the surface of SpeA and form a major hydrophobic contact with the HLA-DR receptor molecule. Mutation of the single residue leucine 42 in SpeA to the charged amino acid side chain of arginine is predicted to disrupt

this major contact with the receptor molecule, resulting in a significant reduction in DR1 binding. This mutant molecule should therefore have lost the toxin attributes of the wild-type molecule. Mutations of SpeA at amino acid position 42 (e.g. L42R or L42A) resulted in greatly diminished interactions with cell surface HLA-DR, as measured by laser fluorescence-activated flow cytometry and FITC-labeled rabbit anti-SpeA antibody (affinity purified). Human T-cell proliferation in response to these mutants was next assessed by [³H]thymidine incorporation, using a 12 h pulse with label and harvesting cells after 60 h of culture. Mutations of SpeA at amino acid position 42 (L42R or L42A) resulted in greatly diminished activation of human lymphocytes (Figure 10). Although alanine or arginine substitutions of L42 were indistinguishable by MHC class II binding, arginine substitution (L42R) resulted in the greatest attenuation of T-cell responses.

20 **EXAMPLE 14**

Design of the SpeA-SpeB fusion antigen/vaccine

The vast majority of *Streptococcus pyogenes* isolates express an extracellular cysteine protease historically termed streptococcal pyrogenic exotoxin B (SpeB). The protease is an important colonization and pathogenicity factor (Kuo, C.-F. et al., 1998, Infect. Immun. 66: 3931-35). However, co-purification of contaminant streptococcal proteins with SpeB led to the erroneous conclusion that the protease was a superantigen. Several potential host substrates are known. For example, the purified SpeB cleaves interleukin 1 precursor protein to produce active interleukin 1 and also cleaves the extracellular matrix proteins fibronectin and vitronectin (Kapur, V. et al. 1993, Microb. Path. 15: 327-346; Kapur, V., et

al.,1993, Proc. Natl. Acad. Sci. U.S.A. 90: 7676-80). The ubiquitous expression of SpeB by *S. pyogenes* and the conserved nature of the antigenic determinants recognized by antibodies are noteworthy features.

- 5 Although multiple alleles exist, polyclonal antisera generated against one SpeB allelic product reacts with SpeB from all *S. pyogenes* M1 serotypes examined (Proc. Natl. Acad. Sci. U.S.A. 90: 7676-80). Based on analysis of the catalytic site structure from
- 10 crystallographic data (T. F. Kagawa, et al., 2000, Proc. Natl. Acad. Sci. USA 97:2235-2240) mutation of active-site residues, for example cysteine at position 47 or histidine at position 340, inactivates proteolytic activity (T. F. Kagawa et al. *supra*;
- 15 Gubba, S.et al., 2000, Infect Immun. 68:3716-9). A mutant, catalytically inactive SpeB (SpeB C47S) was used as a fusion partner with mutant SpeA (SpeA L42R).

- The wild-type SpeB zymogen, cloned from a clinical isolate of GAS, was truncated by PCR cloning
- 20 to produce the mature protein minus the noncatalytic prosegment domain. An additional construct was designed to incorporate the prosegment in the final SpeA-B fusion. Because of solubility problems, only the SpeB minus the prosegment was used for support
 - 25 data. A mutant, catalytically inactive SpeB was constructed by site specific mutagenesis of the DNA coding sequence, altering cysteine at amino acid position 47 to serine. This conservative change maintains the approximate dimensions of the active-
 - 30 site side chain but prevents proteolytic activity. SpeB C47S) was used as a fusion partner with mutant SpeA (SpeA L42R). A pfu DNA polymerase was used for all PCR reactions to lessen the likelihood of
 - 35 introducing spurious mutations common with lower fidelity polymerases, e.g. *taq*. For cloning, the SpeA

(L43R) gene was used as a PCR template and primers 1 (SEQ ID NO:19) and 2 (SEQ ID NO:20) were used to prepare a double-stranded sequence overlapping with SpeB(C47S). A separate PCR reaction using primers 3 (SEQ ID NO:21) and 4 (SEQ ID NO:22) and SpeB (C47S) gene insert was performed to generate a double-stranded DNA fragment overlapping with SpeA (L42R). The PCR fragments were purified by agarose gel electrophoresis and mixed together for a final PCR reaction using primers 1 and 4, to create the full-length gene fusion of SpeA (L42R)-SpeB (C47S). This full-length fragment was blunt-end cloned into the vector pT7Blue (Novagen) and sequence confirmed (SEQ ID NO: 23). The SpeA (L42R)-SpeB (C47S) fusion gene was then subcloned into pET24b(+) for expression in *E. coli* BL21 host strains. The SpeB clone, prosegment plus mature polypeptide is presented in SEQ ID NO:24. The mature SpeB polypeptide used for the SpeA-SpeB fusion is identified in SEQ ID NO:25. The SpeA (L42R) used for the SpeA-SpeB fusion is identified in SEQ ID NO:26. The amino acid sequence of the SpeA-SpeB fusion is identified in SEQ ID NO:27. SEQ ID NO:28-31 identify primers used in the preparation of the SpeA-SpeB fusion, where SpeB prosegment and mature protein were fused with SpeA.

The potential advantages to this fusion construct above the non-fused SpeA mutant are: better activation of immune responses, immune protection against a second virulence factor, cost savings and simplification of product production. The predicted 54 kDa protein was detected by polyacrylamide gel electrophoresis and Coomassie Blue staining. Antibodies specific for either SpeA or SpeB both detected the SpeA L42R-SpeB C47S fusion protein by Western blot analysis.

EXAMPLE 15Mouse antibody response to SpeA L42R and SpeA-B fusion constructs.

Because only minor changes have been introduced into the final protein product,

- 5 maximum antigenicity is maintained. Immune recognition of the SpeA mutants was next examined in an LPS-dependent, murine toxicity model previously described (Stiles, B.G., 1993, *Infect. Immun.* 61:5333). BALB/c mice (female, 20 grams average
- 10 weight; NCI) were vaccinated three times with 10 µg of each construct, allowing two weeks between injections. Sera from each experimental group (n=5) were pooled for measurement of specific antibodies. Data shown in Figure 11 are antigen-specific antibodies (ELISA
- 15 units) present in a 1:100,000 dilution of pooled sera from mice vaccinated with SpeA L42R, SpeA-B fusion or adjuvant only. BALB/c mice were vaccinated three times with 10 µg of each construct, allowing two weeks between injections. Vaccination with either SpeA L42R
- 20 or the SpeA-B fusion produced high antibody titers. As anticipated, antibodies from SpeA L42R vaccination only recognized SpeA, whereas, antibodies from the SpeA-B fusion vaccinated mice recognized both SpeA and SpeB. Although these data confirmed the potent
- 25 immunogenicity of the SpeA constructs, the inbred mouse was an inadequate model to demonstrate protective immunity. Within reasonable physiological limits, wild-type SpeA was not lethal for several inbred mouse strains examined. Therefore, a
- 30 transgenic model was used consisting of mice (H-2^b background) expressing human CD4 and HLA-DQ8 (Taneja, V., and C. S. David. 1999, *Immunol Rev* 169:67; Nabozny, G. H., et al., 1996, *J Exp Med* 183:27). With these transgenic mice SpeA wild-type was lethal at

relatively low concentrations, and the SpeA mutant constructs were also highly immunogenic. HLA-DQ is structurally very similar to HLA-DR, although crystallographic data were not available for the previous molecular modeling studies used for designing the mutant superantigen. Proliferative responses were examined using mononuclear cells isolated from spleens of transgenic mice expressing HLA-DR3, HLA-DQ8 or HLA-DR2 β /IE α , or non-transgenic BALB/c mice and human peripheral blood (Figure 12). These *in vitro* responses of the HLA-DQ+ mice were very similar to results obtained with human mononuclear cells. BALB/c or hemi-transgenic mice in which the mouse IE α was paired with the human HLA-DR2 β subunit required greater amounts of wild-type SpeA to produce a level of proliferation equivalent to the HLA-DQ8 transgenes. Non-vaccinated HLA-DQ8 mice were very sensitive to SpeA challenges, whereas, vaccination with SpeA L42R or the SpeA-B construct fully protected HLA-DQ8 transgenic mice from challenge with the same amount of wild-type SpeA.

Table 13: SpeA Vaccination and Immune Protection: HLA-DQ8/human CD4 Transgenic Mice

Vaccination ¹	Challenge Survival ²
SpeA L42R	100%
SpeA-B fusion	100%
Adjuvant only control 3 doses	0%

¹Vaccinations with 10 μ g L30R, L30A in adjuvant (Alhydrogel) or adjuvant only per mouse 0,2 and 4 weeks (3 dose),

- ²SpeA wild-type i.p. dose 5 LD₅₀ per mouse 2 weeks after last vaccination. Percent survivors by 72 hours. 5 mice per group SpeA L42R and adjuvant only control; 4 mice for SpeA-B fusion vaccination. Experiments involving SpeA L42R were performed twice (n=5 mice per group) with identical results; experiments involving SpeA-B fusion vaccine was performed once.

EXAMPLE 16

Design of altered superantigen toxin vaccine, SEC45

- 10 For Staphylococcal enterotoxin C1 (SEC1), the leucine at position 45 was changed to lysine (SEC45). This mutation is anticipated to prevent SEC1 from interacting with the MHC class II receptor by sterically blocking the hydrophobic loop (centered
- 15 around leucine 45) from binding to the alpha chain of the receptor. SEC1 is more closely homologous to SEB than SEA or the other superantigen toxins. The presence of zinc in SEC1 may impart additional binding characteristics that allow, in some cases, this
- 20 superantigen toxin to bind to T-cell antigen receptors without the required MHC class II molecule interactions. To circumvent the binding to T-cell antigen receptors, mutations of SEC1 residues N23 (changed to alanine), V91 (changed to lysine) are
- 25 being performed.

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